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Physiological Zoölogy

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No. 1

THE ELECTRIC CHARGE OF PROTOPLASMIC COLLOIDS¹

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IT IS of the very nature of colloidal systems that the individual particles or micellae of any colloidal dispersion remain separate from each other because of their electric charge. If the charge is neutralized, the particles tend to come together, and flocculation usually results. Obviously, for the understanding of the behavior of any colloidal dispersion it is a matter of prime importance to know whether the charge on the individual particles or micellae is positive or negative. In the case of the protoplasmic colloid, there is, as yet, no certain information concerning the sign of the electric charge. Ten years ago, in a summary of the information then available, one of us concluded that the charge on the micellae of the protoplasm in the interior of a typical cell was positive (Heilbrunn, 1928, chap. x). Since then, a paper of Sen has aroused wide interest and has been often quoted. Indeed, in their review of electrokinetic methods as related to biology and medicine, Abramson and Moyer (1937) copy almost all of Sen's data and comment favorably upon it. As a result of experiments on the petiole hair cells of *Urtica dioica* (stinging nettle) and on the root hairs of the aquatic plant *Azolla pinnata*, Sen concluded that the protoplasmic particles in these cells bore a negative charge.

Actually, in view of the importance of the question, the literature concerning the electric charge of the protoplasmic colloids in the interior of a living cell is very scanty. There is obviously a need for further work. In this paper we shall report observations on amoeba protoplasm, as well as observations on the protoplasm of the water plant *Elodea*. We have also reinvestigated the root hairs of *A. pinnata*, already studied by Sen.

EXPERIMENTS ON AMOEBA

When an ordinary amoeba, say an *Amoeba proteus*, is placed in an electric field so that an electric current flows through it, the amoeba moves directly and consistently toward the cathode. This is an old observation, due originally to Verworn; and it has been repeated by various other workers (for literature references see Mast, 1931, and Hahnert 1932). In order to be effective, the electric current must have a certain strength; and, on the other hand, it cannot be too strong, for with currents of too great amperage the

¹ This paper was supported by a grant from the Radiation Committee of the National Research Council. It is planned to follow this work with a study of the effects of radiation on the electric charge of protoplasmic colloids.

amoeba breaks at its anodal side and dies. As the amoeba moves toward the cathode, the granules and crystals in its interior move rapidly in a cathodal direction.

Since Verworn's original observation investigators have expressed a wide variety of opinions as to the mechanism of the galvanotropic response of the amoeba. Mast and Hahnert, in the papers cited, summarize these opinions, and they also offer suggestions of their own.

Our interest in the galvanotropic response of amoebae has been in the light it might throw on the sign of the charge on the protoplasmic colloids in the interior of the amoeba cell. The charge on granules and crystals within the amoeba is presumably conditioned largely by the charge on the protein or other colloidal micellae in which the granules or crystals are immersed. The very fact that the granules move in a cathodal direction seems to indicate that their charge can scarcely be negative. However, the granules and crystals move in a flowing mass of protoplasm, and this flow may be due entirely to some change in the boundary of the cell. Thus, if the cathodal edge of the cell were to move toward the cathode, this would pull granules and protoplasm in the same direction. Essentially, there are two possibilities. The cathodic movement of the amoeba may be due to a cataphoretic movement of granules and protoplasmic particles which push the cortex before it (and perhaps also liquefy it), or the cathodic movement of the amoeba as a whole may be due to a primary effect on the cortex or limiting membrane at the cathodal boundary of the cell.

To decide between these two possibilities is not easy. It is possible to imbed amoebae in rather stiff gelatin gels, under conditions which prevent any marked change in shape of the cell. A 10 per cent solution of gelatin is prepared, and this is then cooled rapidly. Before it stiffens, amoebae are dropped into it. The gelatin then hardens, and the amoebae are then completely surrounded by a solid gel which prevents amoeboid movement. Electric currents were passed through such imbedded amoebae, and in one or two experiments it was thought that a movement of granules toward the cathode could be observed. However, in repetitions of this experiment, it was usually impossible to obtain any satisfactory result, either because the amoeba was not completely immobilized by the gelatin or because in other instances it seems to have suffered injury.

Another type of experiment produced much more decisive results. It was reasoned that, if the charge on the protoplasmic colloids within the amoeba was positive, then it should not be difficult to neutralize or reverse this charge by the simple expedient of alkalizing the protoplasm. As is well known from the experiments of Jacobs (1922), ammonium salts cause an entrance of ammonium hydroxide into the cell. Protoplasm can also be alkalized by immersion of cells in solutions of ammonium hydroxide. If now, amoebae are immersed in solutions of ammonium salts or in dilute solutions of ammonium hydroxide, and an electric current is then run through the amoebae, we have a way of determining whether or not the movement toward the cathode as it occurs normally is due to a cataphoretic movement of positively charged granules and protoplasmic micellae. For, if the protoplasmic granules are normally positively charged, then addition of OH ion should decrease or even neutralize this charge, and the granules should move less rapidly or even move in the opposite direction.

Actual experiments showed that this expectation was realized. When specimens of *A. proteus* were immersed in dilute ammonium chloride solutions and then subjected to an electric current, in 48 cases out of a total number of 153, the amoebae did actually move to the anode instead of to the cathode. This is shown in Table 1. Such an anodal

migration of amoebae never occurs normally, as is evidenced by the control experiments with 104 amoebae in spring water. As a matter of fact, in the numerous experiments we have done on the effect of electric current on normal amoebae, we have never seen an amoeba move toward the anode if the current was strong enough to have any effect at all on the direction of movement. Ammonium hydroxide solutions (at a pH of 8.3-9.0) also tend to cause a reversal of the normal galvanotropic response.

The simplest explanation of the galvanotropic reversal following alkalization of the protoplasm is to assume that a normally positive charge of protoplasmic granules is changed to a negative charge. Such a reversal of charge does not occur in all specimens; and, as a matter of fact, it can barely be produced without resultant death of the amoebae. In some cells the charge is apparently neutralized, so that no directed movement occurs at all; in other cases the charge is not quite neutralized and the amoeba

TABLE 1

EFFECT OF AMMONIUM CHLORIDE SOLUTIONS ON THE DIRECTION OF MOVEMENT OF *Amoeba proteus* EXPOSED TO AN ELECTRIC CURRENT

I. CONTROL

Of 104 amoebae in culture fluid, 104 (100%) move to the cathode

II. IN NH_4Cl

Molarity of NH_4Cl	Number of Amoebae Tested	To Anode	To Cathode	Indefinite
0.0166.....	94	38	29	27
0.0142.....	35	16	12	7
0.0133.....	24	12	7	5
Total.....	153	66 (43.1%)	48 (31.3%)	39 (25.5%)

moves slowly toward the cathode. It should also be pointed out that, although these experiments were successful with *A. proteus*, they were not successful with the related species, *Amoeba dubia*. Apparently in the latter form it is not so easy to neutralize the charge on the granules without causing death.

Alkalinization of the protoplasm can apparently be produced in other ways than by immersion of amoebae in ammonium hydroxide or ammonium chloride. The acidity of protoplasm is maintained by the constant production of carbon dioxide, which within the cell is in the form of carbonic acid. This carbonic acid is apparently an important factor in determining the intracellular pH. Thus, in earlier experiments, in which it was shown that alkalinization caused a release of free fat in protoplasm, it was found that an excess of carbon dioxide prevented this release, no doubt because it tended to acidify the protoplasm (Heilbrunn, 1936). Clearly then, any agents which tend to prevent carbon dioxide production (or which tend to remove carbon dioxide from the protoplasm) should tend to alkalinize the protoplasm, and they should therefore have an effect similar to that produced by the entrance of alkali within the cell. Now, it is a well-known fact that, in relatively high concentration, fat solvent anesthetics do cause a pronounced decrease in cellular respiration (for a review of the earlier literature in this field see Winterstein, 1926; references to more recent papers are given by Jowett,

1938). When amoebae are immersed in high concentrations of fat-solvent anesthetics, it is sometimes possible to demonstrate a reversal of charge in the protoplasm. Thus, when amoebae were placed in a 4 per cent ether solution and observed within a few minutes, we were occasionally able to observe a movement of protoplasmic particles and of pseudopodia toward the anode. By the time the observations were made, the concentration of ether was somewhat less than 4 per cent, owing to evaporation of the ether. Similarly, a 2 per cent ethyl urethane solution often causes such a reversal of charge, whereas 0.5 per cent and 1 per cent solutions are ineffective. This effect of high concentrations of fat solvents is interpreted as being primarily due to alkalization.

Of course, it is possible that there are other explanations which might also account for the reversal of galvanotropism in amoeba following alkalization. However, it should be noted that, when amoebae are immersed in ammonium chloride solutions, there is no change in the pH at the cell surface but only a change in the interior pH. Moreover, in ammonium hydroxide solutions with increase in alkalinity the surface membrane of the amoeba should become more negatively charged than ever. This would tend to increase any electroendosmotic flow toward the cathode. If, then, one assumes that galvanotropic response in amoeba is due to an electroendosmotic flow of water toward the cathode as a result of a negatively charged plasma membrane, this flow would be greater in ammonium hydroxide solutions; and on the basis of this theory one should expect a more rapid movement toward the cathode in such solutions. The simplest explanation to account for reversal of galvanotropism with alkalization of the protoplasm is to assume that the original positive charge of the protoplasmic particles is changed to a negative charge. This was the prediction made before the experiment was tried, and the results serve to bear out the prediction. As the granules move either toward cathode or anode, they must tend to break down the thixotropic gel on the side toward which they move. That the cortex of the amoeba is a thixotropic gel is shown by the experiments of Angerer (1936). If this gel is liquefied in any local region, such a region becomes pushed out to form an advancing pseudopod. At any rate, this is the usual interpretation given by modern theories of amoeboid movement.

It seems clear that our experiments on amoeba lend support to the view that the protoplasm in the interior of the amoeba is a positively charged colloid.

EXPERIMENTS ON *Elodea*

In experiments with amoeba the changes in shape of the cell introduce difficulties. Plant cells typically are inclosed by a rigid cell wall, so that the boundaries of the cell remain in a constant position. However, in dealing with plant material it is not always a simple matter to determine when the protoplasm is injured or dead. In many plant cells the protoplasm constantly streams; and this streaming, or cyclosis, ceases on violent injury or death. Accordingly, such cells with streaming protoplasm offer advantages for the solution of our problem, for it is possible to adjust the strength of the electric current so that it will cause an effect and yet not produce death.

The well-known water plant, *Elodea canadensis*, has been widely used in studies of protoplasm and is readily available. The protoplasm in the leaf cells of this plant may stream when the leaves are on the plant. This streaming tends to become more rapid, and it is of more regular occurrence after the leaves have been cut away. In our experiments leaves were cut from the stem and allowed to remain in water for at least 1 hour before they were studied. The current was led to the leaves through agar bridges from

nonpolarizable zinc-zinc sulphate electrodes. The electrodes were connected with a 110-volt circuit, and the amperage was then varied by inserting suitable resistance.

In currents not quite strong enough to cause stoppage of protoplasmic streaming the rate of movement of the chloroplasts is affected by the electric current. This was determined by noting the speed of the chloroplasts as they moved around the cell. In the one direction they traveled toward the cathode; and in the other, toward the anode. We would have preferred to measure the speed of the tiny protoplasmic granules rather than the speed of the chloroplasts, but such measurement is very tedious and technically well-nigh impossible for a long series of experiments. As a matter of fact, the measurement of the speed of chloroplasts is not too easy, for one must make a series of measurements for the same cell before and after the application of the electric current. It is essential to select a cell for measurement which does not have too many chloroplasts, for when these structures are very numerous, they tend to get in each other's way. The result is that the movement may become jerky and irregular, and measurements under these conditions are not very satisfactory.

In measuring the movement of chloroplasts rather than protoplasmic granules, we were at a disadvantage. Although the electric charge of the chloroplast is in large measure determined by the charge of the micellae of the protoplasm in which it is bathed, one cannot deny that the essential nature of the chloroplast itself may be a factor. Our results show the sign of the electric charge at the surface of the chloroplast, and it is something of an assumption to suppose that this charge is the same as that of the rest of the protoplasm. At the worst, we do obtain information concerning the chloroplast charge.

The experiments were performed in the following manner. A detached *Elodea* leaf, or a section of such a leaf, was placed in spring water between two agar bridges leading to the electrodes. The leaf was oriented so that the current flow was in a direction parallel to the midrib; in other words, the current flowed through the length of the cells of the leaf rather than across them. With the leaf in position between the agar bridges, a favorable cell was chosen. When this cell was viewed in optical section, the chloroplasts could be observed moving in a given direction along one of the long walls and in an opposite direction along the opposite wall. Their speed along each of these walls was then determined with the aid of an ocular micrometer eyepiece and a stop watch. Usually, the scale of the ocular micrometer could be moved first to a position over one wall of the cell and then over the opposite wall by the simple expedient of rotating the ocular of the microscope. Typically, ten measurements were made on each side, and these were averaged. In general in these control experiments the average speed of chloroplast movement on the two sides of the cell was approximately the same, and only rarely did the difference in rate of speed exceed a value of 15 per cent. Once we were sure that the cell was behaving with reasonable normality and that the speed of chloroplast movement on opposite walls was not widely different, the electric current was applied. During the time that the electric current was flowing, the chloroplasts were moving with the current on one side of the cell and against the current on the opposite side. With the electric current flowing, measurements of the rate of movement of the chloroplasts on the two sides of the cell were made with the same technique as that used before the electric current was turned on. In many cases it was obvious that the electric current was having an effect, for the difference in rate of chloroplast movement on the two sides of the cell was often much greater than 15 per cent. Not infrequently, differences as great as 30

or 40 per cent were obtained. The results of these experiments are given in Table 2. They were very disappointing. It had been hoped that the speed of the chloroplasts

TABLE 2
EFFECT OF AN ELECTRIC CURRENT ON THE SPEED OF CHLOROPLASTS IN *Elodea* CELLS

No OF EXPT	RATE OF STREAMING IN CONTROL			RATE OF STREAMING DURING PASSAGE OF CURRENT			STRENGTH OF ELECTRIC CURRENT (IN MILLI- AMPRES)
	To Right (μ /Sec)	To Left (μ /Sec)	Percentage Difference	Toward Anode (μ /Sec)	Toward Cathode (μ /Sec)	Percentage Difference	
1	6 5	6 1	6 5	6 1	4 8	27 0	5 9
2	3 7	3 6	2 7	3 5	4 9	40 0	2 0
3	7 6	7 6		10 4	6 9	50 7	3 0
4	5 2	5 7	9 6	5 7	4 1	39 0	2 0
5	5 9	5 5	7 2	4 6	6 3	36 9	3 0
6	5 5	6 0	9 0	6 9	4 9	40 8	6 0
7	5 2	4 5	15 5	3 8	5 0	31 5	4 0
8	5 3	5 8	9 4	6 5	5 6	16 0	4 0
9	4 1	4 3	4 8	4 6	3 3	39 3	5 0
10	2 9	2 6	1 1	2 9	3 8	31 0	2 0
11	5 3	4 6	15 2	3 8	3 7	2 7	2 0
12	5 9	6 0	1 6	6 3	4 3	46 5	3 0
13	4 0	4 0		2 7	3 3	22 2	2 0
14	5 7	6 1	7 0	4 4	4 1	7 3	2 0
15	3 8	4 0	5 2	4 2	3 4	23 5	2 0
16	5 6	6 3	12 5	6 5	4 1	58 5	2 0
17	5 6	5 9	5 3	6 1	4 5	35 5	1 0
18	6 6	5 8	13 7	6 1	6 9	13 1	1 0
19	5 8	6 5	12 0	7 4	5 8	27 5	2 0
20	5 1	4 9	4 0	6 3	4 9	28 5	1 0
21	3 9	4 0	2 5	4 5	3 7	21 6	0 5
22	2 5	2 5		3 2	2 2	45 4	2 0
23	4 3	3 9	10 2	3 6	5 0	38 8	1 25
24	5 8	5 4	7 4	5 6	4 0	40 0	6 7
25	6 1	5 9	3 3	3 9	2 4	62 5	6 9
26	4 0	4 1	2 5	3 2	4 6	43 7	5 4
27	3 2	3 3	3 1	2 9	2 4	20 8	2 5
28	2 8	2 9	3 5	2 5	2 8	12 0	2 0
29	6 0	6 5	8 3	6 4	8 2	28 1	4 0
30	6 0	7 4	23 3	6 0	3 4	76 4	3 0
31	4 6	4 7	2 1	3 9	3 1	25 7	1 0
32	7 8	7 7	1 2	8 2	7 0	17 0	0 1
33	7 3	7 0	4 2	5 8	8 0	37 9	0 15
34	6 7	7 1	5 9	5 9	9 2	55 9	0 15
35	4 6	4 2	9 5	5 4	4 7	14 8	0 1
36	5 4	5 6	3 7	3 2	4 6	43 7	0 125
37	6 4	6 8	6 2	5 9	7 1	20 3	0 125
38	5 7	5 5	3 6	4 9	6 4	30 6	
39	5 5	5 4	1 8	6 5	5 9	10 1	0 15
40	6 4	5 6	14 4	4 9	5 8	18 3	0 175

would be greater either to the cathode or to the anode. Actually, in some experiments the speed was greater toward the cathode; in some other cases it was greater toward the anode. Inspection of Table 2 shows sixteen cases of greater speed toward the cathode and twenty-two cases of greater speed toward the anode. To these twenty-two cases

might be added two other cases in which there was a greater speed toward the anode but in which the difference in speed seemed insignificant (less than 10 per cent). The whole experiment might have seemed futile if it were not for the fact that in many cases the electric current appeared to be exerting a very real influence. Thus, in one instance the speed of chloroplasts was 76 per cent greater when the chloroplasts were traveling toward the anode than was the speed when the chloroplasts were traveling toward the cathode. In another case there was a 56 per cent difference in favor of the movement toward the cathode.

Therefore, the results apparently indicate that the chloroplasts are actually charged; but if we are to draw any conclusions, it would seem to be true that they are charged sometimes positively and sometimes negatively. At first we were at a loss to account for this apparent difference in charge in different experiments, and we made a number of efforts to conduct our experiments in such a way as to show that the chloroplasts are always charged either positively or negatively. Thus the experimental technique was varied by cutting the leaves in different ways; but no matter which type of leaf preparation was used, there was no constancy in the charge of the chloroplasts as determined by the relative speed of their movement toward cathode or anode.

Finally, we were led to believe that the chloroplasts might be charged either positively or negatively and that the sign of the charge depended on the intensity of photosynthesis in the cell. This point of view seemed logically plausible, for during photosynthesis carbonic acid is being rapidly removed from the protoplasm, whereas in the absence of photosynthesis the protoplasm can be assumed to be drenched with carbonic acid. The presence of more or less carbon dioxide may have a profound influence on the hydrogen ion concentration of the protoplasm. This point has already been discussed in relation to the experiments with amoeba. Accordingly, we might expect that during active photosynthesis the protoplasm in general, and the surface of the chloroplast in particular, would tend to be more negatively charged, owing to the absence of acid carbon dioxide. On the other hand, when photosynthesis is not occurring, the presence of carbon dioxide would tend to make the charge on protoplasmic micellae and chloroplasts positive.

Thus, if one could eliminate photosynthesis, the charge on the particles within the protoplasm might always be positive. For a time we tried to make measurements of chloroplast speed in almost complete absence of light. This did not prove practicable. Indeed, it is difficult enough to obtain measurements even with the best illumination possible. We looked then for ways of inhibiting photosynthesis in the light. Years ago, Claude Bernard showed that dilute solutions of fat-solvent anesthetics could prevent photosynthetic activity in green plants without having much effect on respiration, and this work has had the support of later experimenters (for literature references see Winterstein, 1926, pp. 138-41). In general, it may be said that there is a rather narrow range of concentration in which fat solvents, such as chloroform, depress photosynthesis without depressing respiration. Using the technique already described, we studied the influence of electric current on chloroplast speed in leaves immersed in dilute solutions of chloroform and of ethyl urethane. We hoped that exposure to these anesthetics would give us uniform results and that in these experiments the chloroplasts would always behave as if positively charged. Again we were disappointed, for, as before, the chloroplasts were sometimes charged positively and sometimes negatively. It is quite possible that we did not select the exact concentration of anesthetic necessary to inhibit photo-

synthesis without changing the rate of respiration, and this may account for our failure to obtain consistent results.

Clearly we needed more information as to the conditions under which photosynthesis might be inhibited in our *Elodea* material in the light. Modern studies of photosynthesis use standard analytical methods for the determination of oxygen liberated or carbon dioxide utilized. In general, however, these newer methods confirm the older observations made simply by counting the number of bubbles of oxygen given off by aquatic plants such as *Elodea*. We were in search of only qualitative information, and for this reason we adopted the simple technique of Sachs for watching the course of photosynthesis. An ordinary funnel was inverted under water, and its tube was suitably covered with a test tube also filled with water. *Elodea* plants were then imprisoned under the funnel. The oxygen bubbles released as a result of photosynthetic activity passed up into the test tube, displacing the water at the upper end of the test tube. From time to time the volume of oxygen gas in the test tube could be measured. With such a simple arrangement we were able to note two sets of conditions in which photosynthesis was inhibited. In the first place, although photosynthesis was very active for the first day during which the *Elodea* plants were imprisoned under the funnel, the rate of photosynthetic activity, as measured by gas production, fell off rapidly, so that after several days there was no increase in the volume of oxygen in the test tube; and at this time, bubbles were no longer given off by the plants. Secondly, after *Elodea* leaves had been kept in the dark for several days, their photosynthetic activity did not return immediately; or, at any rate, in the case of such plants removed from the dark the evolution of oxygen was, for a time, very slow.

Our first experiments were on the leaves of plants which had been imprisoned under funnels for several days. Why photosynthesis should have stopped in these leaves we do not know. The fact remains, however, that it did stop. Measurements of chloroplast speed were then made on these leaves which, while under the funnel, had ceased to show photosynthetic activity. A total of twenty-three series of measurements were made. In every case the chloroplasts showed more rapid movement toward the cathode, indicating that in every case they were charged positively. Although these experiments gave us the result we had hoped to obtain, we were not very content with them, largely because we could not understand why imprisonment under the funnels should cause cessation of photosynthesis. We therefore turned to the second type of condition in which photosynthesis is retarded even though the plant cells are in the light.

Elodea plants were kept in the dark for 6-9 days; the leaves were then cut off and were studied with our usual technique. Photosynthetic activity tends to begin soon after the leaves emerge into the light. Accordingly, it was necessary to make measurements of chloroplast speed as rapidly as possible. Usually the measurements were completed within about a half-hour after exposure to light was begun. Moreover, in making the measurements, light passing through the leaves was cut down to some extent. Before the light entered the leaf it usually passed through a green filter, and this served to cut down the intensity of the radiation. The results obtained in experiments with leaves previously kept in the dark are shown in Table 3. They are wholly consistent. In all, the chloroplast speed was measured in twenty-six leaf preparations. In twenty-four of these preparations the chloroplasts moved toward the cathode with a speed significantly greater than that they showed toward the anode. In the other two preparations, one showed no difference in rate of speed toward cathode or anode while the other showed

an increase in rate of speed toward the cathode; but this was not significant, for the difference was not appreciably greater than that shown in the control (before the current was applied). In no single experiment was the chloroplast speed greater toward the anode, so that in 100 per cent of those leaves which gave a clear answer, the speed of the chloroplasts was greater toward the cathode. Obviously, under the conditions of

TABLE 3
EFFECT OF AN ELECTRIC CURRENT ON THE SPEED OF THE CHLOROPLASTS IN *Elodea*
LEAVES WHICH HAVE BEEN KEPT IN THE DARK

No of EXPT	RATE OF STREAMING IN CONTROL			RATE OF STREAMING DURING PASSAGE OF CURRENT			STRENGTH OF ELECTRIC CURRENT (IN MILLI- AMPERES)
	To Right (μ /Sec)	To Left (μ /Sec)	Percentage Difference	Toward Anode (μ /Sec)	Toward Cathode (μ /Sec)	Percentage Difference	
1	4 5	4 7	4 4	2 9	3 6	24 1	0 175
2	5 4	5 1	5 8	4 3	5 5	27 9	0 175
3	5 0	5 0		4 8	5 9	22 9	0 175
4	4 2	4 3	2 3	3 9	4 9	25 6	0 175
5	5 1	5 1		4 3	6 1	41 8	0 175
6	9 5	8 9	6 7	7 6	10 1	32 8	0 2
7	8 0	7 3	9 5	3 4	5 8	70 5	0 325
8	4 9	5 2	6 1	4 5	5 6	24 4	0 9
9	6 3	6 3		5 5	7 6	38 1	0 875
10	5 2	4 9	6 1	4 7	6 3	34 0	0 95
11	3 8	4 0	5 2	3 7	5 1	37 8	0 2
12	4 8	4 4	9 0	4 0	5 9	47 5	0 175
13	3 1	3 2	3 2	2 9	4 0	37 9	0 2
14	3 8	3 8		3 9	4 3	10 2	0 15
15	4 7	4 8	2 1	3 6	4 1	13 8	0 15
16	5 9	5 5	7 2	5 2	5 6	7 6	0 15
17	3 1	3 1		2 8	3 3	17 8	0 2
18	4 8	4 7	2 1	3 8	4 3	13 1	0 2
19	2 9	2 8	3 5	2 8	3 6	28 5	0 2
20	4 3	4 6	6 9	3 9	5 2	33 3	0 2
21	3 1	3 0	3 3	2 4	3 7	54 1	0 2
22	2 8	3 2	14 1	2 8	2 8		0 2
23	3 0	3 5	16 6	2 5	3 1	24 0	0 175
24	3 0	3 4	13 3	2 3	3 5	52 1	0 2
25	3 3	3 2	3 1	2 7	3 4	25 9	0 175
26	4 1	3 8	7 8	4 0	5 7	42 5	0 175

the experiment, the chloroplasts bore a positive charge. It should be emphasized once more that these measurements must be made reasonably soon after the leaves emerge into the light. In a number of cases the cells were studied again after they had remained in the light for a half-hour or longer. Under these conditions the chloroplasts sometimes showed a positive, sometimes a negative, charge.

It may, with reasonable safety, be concluded that in the dark, or under conditions which tend to inhibit photosynthesis, the electric charge on the chloroplasts, and presumably also on the protoplasmic micellae of the *Elodea* cell, is positive. There is thus additional evidence that the normal charge of the protoplasmic colloid is positive. It is a matter of considerable interest that this charge may be reversed in sign by photosynthesis.

EXPERIMENTS ON *Azolla*

The results thus far obtained have been wholly consistent with the thesis that the colloidal particles of protoplasm bear a positive charge. How, then, are we to interpret the results of Sen, already mentioned? It will be remembered that Sen investigated the petiole hair cells of *U. dioica* and the root-hair cells of *A. pinnata*. It is the results with *Azolla* cells that are especially interesting, for in these cells Sen was able to give quantitative figures for the cataphoretic movement of particles within the cells. Abramson and Moyer thought these figures so important that they recopied them in their 1937 paper.

In his discussion Sen states that "the particles very near the cell walls scarcely moved, and, if at all, toward the cathode." Particles farther away from the cell walls moved toward the anode, and those about midway between the cell walls showed the maximum velocity. In thinking over these observations it occurred to us that the particles which Sen observed moving toward the anode might be in the cell vacuole rather than in the cell protoplasm. Indeed, a statement to this effect was made in a recent book (Heilbrunn, 1937, p. 77). However, Sen states clearly that he was studying protoplasmic particles. It was thought worth while, therefore, to examine the root-hair cells of *Azolla* in order to determine whether or not the particles studied by Sen were really protoplasmic or not.

The root hair of *Azolla*, viewed under dark-field illumination, is a beautiful object. The granules in the center of the cell are in active Brownian movement. As soon as they are subjected to an electric current, they show a rapid cataphoresis. When the direction of the current is reversed, the granules also reverse their movement. In every case they move toward the anode. It is thus a simple matter to confirm Sen's observations. However, there is a question as to whether the granules which show such beautiful Brownian movement and which move so readily toward the anode are really in the protoplasm. Sen states that the *Azolla* root hair is "practically a cellulose capillary of uniform diameter filled with transparent protoplasm." But if one examines Sen's figures, especially his Figure 1, the cell appears like a typical root-hair cell with a narrow layer of protoplasm surrounding a central vacuole which occupies the major portion of the cell. Is this the true structure? In order to settle this point, root hairs were stained with neutral red. In such stained cells the red color was largely confined to a narrow layer beneath the cell wall. However, this could hardly be regarded as conclusive proof that the center of the cell is a vacuole. Much better evidence is given by experiments in which the cells were treated with 70 or 95 per cent alcohol. After *Azolla* preparations had been thoroughly fixed in such alcoholic solutions for 4-12 hours, the granules in the center of the cell retained their Brownian movement and moved toward the anode, just as they did in the living preparations. Obviously, following fixation with alcohol, Brownian movement in protoplasm ceases, and cataphoretic movement of protoplasmic particles would also be inhibited.

There is this difference between the behavior of the *Azolla* cells fixed in alcohol and living *Azolla* cells. When an electric current is run through the living cells, the protoplasmic layer beneath the cell wall seems to grow thicker, and it apparently becomes sticky. The result is that the actively moving particles in the center of the cell after a time cease Brownian movement and no longer move under the influence of the electric current. However, when an electric current is run through cells fixed in alcohol, the granules in the center of the cell continue to respond to the electric current for hours. Sometimes after a lengthy exposure the granules become aggregated into balls whose

diameter is perhaps two-thirds of the entire cell. Under the influence of the electric current these balls can be seen to move back and forth as the direction of the electric current is changed. Like the granules of which they are composed, the balls move rapidly toward the anode. Anyone observing these balls rolling through the central region of the *Azolla* root hair could scarcely doubt but that this is a typical root hair and possesses a well-developed vacuole.

It is thus clear than Sen's observations on the granules in the center of *Azolla* cells were made on particles within the cell vacuole. They thus offer no information concerning the charge on protoplasmic particles. It will be remembered that for particles near the cell wall, thus presumably in the protoplasm, Sen observed a movement toward the cathode.

DISCUSSION

Our results with the amoeba and with *Elodea* bear out the opinion expressed earlier (Heilbrunn, 1928) that the charge of the particles of the protoplasmic colloid is typically positive. This charge is apparently dependent on the concentration of carbon dioxide present in the protoplasm. If this carbon dioxide is reduced in amount, as it may be in photosynthesis, the sign of the charge may be reversed.

In addition to the data which we have presented, there is another observation which indicates a positive charge of protoplasmic colloids. Churney and Klein (1937) passed electric currents through the large salivary gland cells of the larva of the fly *Sciara*. As a result of the passage of the electric current the chromatin within the nucleus moved toward the anode, but the nucleus as a whole moved toward the cathode. Although it was unknown to Churney and Klein at the time of their experiments, a similar observation was made by Dahlgren in 1915. He passed electric currents through nerve cells of the electric ray (*Torpedo ocellata*) and observed a movement of the nuclei toward the cathode. Dahlgren's observations are illustrated in his Figures 19 and 20. He was not interested in determining the charge on protoplasmic colloids, and he makes no mention of these experiments in the summary of his paper. It seems possible that the cells of electric fishes would constitute favorable material for the study of cataphoretic effects, for the protoplasm of these cells is no doubt accustomed to the passage of an electric current and would not be so readily killed as is the protoplasm of a typical cell.

A cell nucleus might well be assumed to have its surface impregnated with the colloids of the cytoplasm. Hence, presumably its surface would bear the same charge as that of the protoplasmic micellae. It is consistent with our thesis, therefore, that Churney and Klein, and also Dahlgren, should observe a movement of the nucleus as a whole toward the cathode.

In undertaking our experiments with the amoeba, our primary purpose was to investigate the charge on the micellae of the protoplasm. Viewed from another standpoint, however, our results can be taken to serve as an explanation of the galvanotropic behavior of the amoeba. Indeed, they constitute a rather complete mechanical explanation of this galvanotropism. Moreover, it is possible to interpret reversal of the tropistic response as caused by fat solvents.

SUMMARY

1. The movement of an amoeba toward the cathode is due to the positive charge of its protoplasmic particles. This is indicated by the fact that, when the protoplasm is

made alkaline by treatment with NH_4OH or NH_4Cl , the particles within the amoeba and the amoeba as a whole may often be caused to migrate toward the anode.

2. The charge on protoplasmic micellae is thus dependent on the pH of the cell interior.

3. When amoebae are placed in concentrated solutions of fat solvents, they tend to move toward the anode, indicating that the charge on their protoplasmic colloids has been reversed. This is believed to be due to an effect of these concentrated solutions of fat solvents on the rate of respiration. With decrease in carbon dioxide in the protoplasm the colloidal particles in the protoplasm tend to assume a negative charge.

4. When an electric current is sent through the streaming protoplasm of an *Elodea* cell, the chloroplasts sometimes tend to move more rapidly toward the cathode; sometimes they move more rapidly toward the anode. The results are taken to mean that the charge on the chloroplasts may be either positive or negative.

5. Under conditions which tend to inhibit or retard photosynthesis, the chloroplasts of *Elodea* were found always to move more rapidly toward the cathode.

6. The conclusion to be drawn is that the typical charge of the micellae of the protoplasmic colloid is positive. This positive charge may be reversed by suitable alkalinization of the protoplasm. Such alkalinization may be produced (a) by the direct addition of alkali to the protoplasm; (b) by reduction in the amount of carbon dioxide in the protoplasm as a sequence of a lowered rate of respiration; (c) by reduction in the amount of carbon dioxide in the cell as a result of photosynthesis.

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THE VOLUMES OCCUPIED BY THE FORMED CYTOPLASMIC COMPONENTS IN MARINE EGGS¹

(One plate)

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THE visible formed components in living cells, including the oil drops, yolk spheres, pigment granules, mitochondria, Golgi bodies, and the like, constitute, together with the hyaline ground substance, a heterogeneous coarse suspension. While it is not possible to characterize any of these elements as "living," in contradistinction to "lifeless," constituents of the protoplasm, nevertheless there is reason to conclude that, of all the cell constituents, the clear hyaloplasm is the most constant and the most active, and may perhaps be regarded as forming the fundamental basis of the protoplasmic system (Wilson, 1925, p. 78).

Information concerning the relative quantities of the formed components in different types of cells must be considered in the investigation of numerous problems. The direct chemical analysis of protoplasm may be first mentioned. Chemical analyses of protoplasm are not directly comparable, since investigators have made use of cells of varying types, containing unknown proportions of these formed components, some of which may perhaps be considered as inert cell products rather than as active constituents of living substance. Indeed, if the chemical composition of protoplasm in many different cells or organisms should happen to be the same, the varying proportions of inert products also included in the analyses would obscure this identity.

Information regarding the relative volumes of visible formed components has been found useful in the solution of other problems. Heilbrunn (1926) made use of the calculated relative volumes of yolk and pigment granules in his determination of the viscosity of the *Arbacia* egg. E. N. Harvey (1932) calculated the relative volumes of the several layers of substances in the centrifuged *Arbacia* egg. These calculations, together with unpublished data of the author for the egg of *Echinarachnius*, were used by Holter (1936) in ascertaining the place of action of the peptidases in these eggs. Holter also calculated the relative volumes of the dispersed substances in the egg of *Chaetopterus*.

The widespread occurrence of intracellular inclusions, such as granules and vacuoles, which frequently take on a color with dyes or which possess natural pigments, makes it necessary to consider them in relation to the pH or rH of protoplasm. Chambers

¹ This study was begun in 1932. A part of the work was performed during the tenure of a National Research Fellowship in the Biological Sciences (1934-35). Measurements on the eggs of *Anisodoris*, *Archidoris*, *Arenicola*, *Cadlina*, *Diaulula*, *Diplodonta*, *Hopkinsia*, *Mytilus*, *Polychoerus*, *Rostanga*, *Strongylocentrotus*, *Triopha*, and *Urechis* were made at the Hopkins Marine Station of Stanford University. All other measurements were made at the Marine Biological Laboratory, Woods Hole, Massachusetts. I am indebted to Dr. W. K. Fisher, director of the Hopkins Marine Station, and to Dr. M. H. Jacobs, director of the Marine Biological Laboratory, for providing facilities for the work; also to Dr. C. V. Taylor for reading the manuscript.

(1929) has already discussed this problem in detail. The separation of the different types of granules by the use of the centrifuge, however, reveals with the greatest delicacy the location of injected or absorbed dyes.

Furthermore, there is an indirect relationship between the relative volumes of formed substances and the osmotic properties of cells. The fate of the water that enters a living cell from a hypotonic solution is not well understood. Some authors maintain that it occupies, for a period of time, discrete vacuoles within the cytoplasm. Few cells behave as perfect osmometers, a fact generally attributed to the presence within most cells of osmotically inactive substances. McCutcheon, Lucké, and Hartline (1931) investigated the osmotic properties of unfertilized *Arbacia punctulata* eggs in sea water of various concentrations. Employing a formula of Hamburger (1898) for estimating the osmotically inert material, they found that, if one takes into consideration a quantity of dispersed substances occupying 7-14 per cent of the egg volume, the product of the volume of the eggs and the osmotic pressure of the external solution is almost constant. In addition, Białasiewicz (1933), likewise applying the principle of Hamburger to the study of unfertilized eggs, found that the volume of the dispersed phase ("espace mort," "Protoplasmaagerüst") were as follows for the eggs of four echinoderms: *Phallusia mamillata*, 19.3 per cent; *Echinus microtuberculatus*, 22.6 per cent; *Paracentrotus lividus*, 25.1-27.0 per cent; *Arbacia pustulosa*, 36.4 per cent. Białasiewicz' results were consistent only for hypertonic solutions, because in hypotonic solutions there was "un gonflement des colloïdes oöplasmiques qui résulte de la dilution des électrolytes dans le liquide intermicellaire." The osmotic properties of the egg may well be influenced by the relative quantities of the formed components. That is, the volume of the dispersed phase may correspond in part to the volume occupied by the cytoplasmic inclusions. Measurements of the volumes occupied by the formed components in the normal egg are preliminary to experiments upon the volumes occupied by these same components in solutions of various osmotic pressures.

Finally, the investigation of the nature of living substance, to ascertain whether it is colloidal or noncolloidal, should make use of the hyaline ground substance only, free of all extraneous material, such as visible granules and vacuoles (Just, 1936). A study of the relative amounts of such formed components in many types of cells should facilitate the selection of material suitable for such an investigation.

METHODS

The separation of the several types of substances in the living egg cells was brought about by means of the centrifuge. Any egg cell subjected to a sufficiently great centrifugal force for an adequately long period showed a stratification of the cell contents. After removal from the centrifuge tubes, the eggs were photographed at right angles to the axis of centrifuging. With the aid of the photographs the volumes of the different strata were calculated. Two methods of calculation were employed. First, the volumes of the spherical segments of known height were obtained by direct geometrical computation. Second, a graph was constructed, plotting height of the spherical segment, as a function of the egg diameter, against segment volume. This resulted in a sigmoid curve, from which volumes of spherical segments of known height could be read off, thus obviating the necessity of individual computations.

The errors involved in the determination of the volumes occupied by the formed components of the egg are many and difficult of evaluation. Errors may arise as a result

of (1) insufficient packing of the granules, owing to (a) employing too low a centrifugal force, (b) centrifuging for too short a time, or (c) slight redistribution of granules before photographing; (2) differences in the diameter of the granules of a given segment, making difficult the estimation of the free space (i.e., hyaloplasm) between the packed granules (26 per cent for incompressible granules of uniform diameter); (3) nonsphericity of the centrifuged egg, owing to the elongation produced by the separation of centripetally directed oil from centrifugally directed yolk, rendering inaccurate the calculation of the volumes of the segments. To these errors should perhaps be added the unknown factors introduced by possible electrokinetic charges upon the cytoplasmic granules and by distortion of the shape of the granules under high centrifugal forces.

Some of the foregoing listed sources of error may be eliminated. It is possible to centrifuge eggs at various magnitudes of centrifugal force for varying periods of time. Providing that the egg does not cytolize or fragment under these conditions, the limit of granule-packing may thereby be determined. It is also possible to observe the rate of redistribution of the granules in the centrifuged eggs and to allow for the amount of redistribution that has occurred at the time the photograph is taken. Unfortunately, it is not possible to estimate the amount of free space between the packed granules other than by comparison of the volumes obtained by varying centrifugal treatments. There is no doubt but that the granules in any one stratum vary greatly in size. Since it is therefore impossible to apply a correction designed for granules of uniform diameter, the measurements are recorded in this paper in terms of the volumes occupied by the several strata of material, without allowing for the quantity of hyaline protoplasm filling the interstices between the granules of these various layers. Nonsphericity of the entire egg may be estimated and allowed for in the calculations of the volumes of the spherical segments.

Since the measurements recorded in the present paper have been accumulated over a period of years, using many different types of centrifuges, it has not always been possible to subject the experimental material to a sufficient range of centrifugal forces to fulfil all these conditions. The maximum centrifugal force employed for each egg species will therefore be included in the data. It is quite probable that the application of increasingly high centrifugal forces to these same species of eggs would yield results of a higher order of accuracy. Some eggs, however, would cytolize under the influence of higher centrifugal forces.

RESULTS

The results are presented in Table 1 in terms of the relative volumes of the centrifuged egg occupied by the spherical segments of the different substances. In column 1 are listed the species of eggs studied; in column 2, the volume, in percentage of total egg volume, of the centripetally located fat or oil zone; in column 3, the volume of the hyaline segment; in column 4, the volume occupied by the "yolk" granules; in column 5, the volume occupied by other types of granules; in column 6, the volume of the germinal vesicle, if present; and in column 7, the maximum centrifugal force employed.

The oil or fat zone is the most centripetally located segment in each centrifuged egg. When only one hyaline zone is present, this segment is just centrifugal to the oil layer, and contains, at the appropriate stage of egg maturation, the germinal vesicle or the nucleus. Frequently the maturation spindle is also thrown into this segment. When two zones of hyaloplasm are present, one is at the position just mentioned and the second is

between the yolk segment and the segment occupied by the heaviest granules of the egg. This condition was found in *Echinarachnius*, *Polychoerus*, and strongly centrifuged *Nereis* eggs. There is a possibility that this second hyaline zone may have resulted from

TABLE 1
RELATIVE VOLUMES, IN PERCENTAGES, OCCUPIED BY THE FORMED COMPONENTS
OF EGGS CENTRIFUGED AT INDICATED FORCES*

SPECIES	RELATIVE VOLUMES (PER CENT)					MAXIMUM CENTRIFUGAL FORCE (TIMES GRAVITY)
	Oil	Hyaline Zone	Yolk	Other Granules	Germinal Vesicle	
<i>Amphitrite ornata</i>	25	38	31	6	6,000
<i>Anisodoris nobilis</i> (fertilized) ..	3	29	68	270,000
<i>Arbacia punctulata</i>	2	45	38	10+5	6,000
<i>Archidoris montereyensis</i> (fertilized) ..	3	30	67	240,000
<i>Arenicola clapedii</i> (fertilized) ..	15	35	50	4,800
<i>Asterias forbesi</i>	3	20	77	6,000
<i>Cadlina flavomaculata</i> (fertilized) ..	3	30	67	265,000
<i>Cerebratulus lacleus</i>	5	52	35	8	6,000
<i>Chaetopterus pergamentaceus</i> ..	7	48	45	6,000
<i>Cumingia tellinoides</i>	10	42	35	13	20,000
<i>Diaulula sandiegensis</i> (fertilized) ..	3	31	66	265,000
<i>Diplodonta orbella</i> (fertilized) ..	25	55	20	150,000
<i>Echinarachnius parma</i>	1	{ I—10 II—10	60	19	6,000
<i>Glycera</i> sp.	8	42	50	6,000
<i>Hopkinsia rosacea</i> (fertilized) ..	2	31	67	100,000
<i>Hydroides hexagonus</i>	10	60	30	6,000
<i>Mytilus californianus</i>	14	55	31	4,800
<i>Nereis limbata</i> (unfertilized) ..	9	33	55	3	6,000
<i>N. limbata</i> (fertilized)	8	{ I—16 II—9	55	9+3	240,000
<i>N. limbata</i> (fertilized)	9	36	55	6,000
<i>Phascolosoma gouldi</i>	13	40	47	6,000
<i>Polychoerus carmelensis</i> (fertilized) ..	8	{ I—15 II—3	60	4+10	100,000
<i>Rostanga pulchra</i> (fertilized) ..	5	20	75	100,000
<i>Sabellaria vulgaris</i>	14	53	33	6,000
<i>Strongylocentrotus purpuratus</i> ..	2	28	70	50,000
<i>Styela partita</i>	4	26	60	6	4	6,000
<i>Triopha carpenteri</i> (fertilized) ..	3	38	59	100,000

* These volumes are not corrected for the hyaloplasm occupying the interstices between the granules. The eggs are unfertilized unless otherwise indicated.

the breakdown (cytolysis) of some of the heavier yolk spheres, although this is unlikely. Where only three zones of stratification are present, the zone of the yolk spheres is centrifugal to hyaline zone I. That is, it is the most centrifugally located segment of the egg. In the yolk segment of certain eggs superficially showing only three layers (oil, hyaline zone, and yolk), there may be distinguished granules of different size or density that have not been completely segregated by centrifuging. The heaviest yolk

granules are usually identical in staining reaction with the erythrophilous (neutral-red staining) granules, and in many cases only by centrifuging stained eggs can the stratum of ordinary yolk granules be distinguished from the stratum of the heavier erythrophilous yolk granules. In the egg of *Asterias forbesi*, for example, staining with neutral red reveals erythrophilous granules at the centrifugal pole of the yolk zone, which are indistinguishable from yolk granules in unstained eggs. In the egg of *Rostanga pulchra* the yolk granules are of two types, colorless and orange, and the orange granules form a fairly distinct segment centripetal to the colorless ones. These pigmented yolk granules give the characteristic color to the egg ribbon. In certain lots of *Phascolosoma* eggs, yolk granules of dark brown and of pale brown were to be distinguished, forming indistinct zones. Where these different types of yolk granules were incompletely separated from each other with the centrifugal technique employed, they were included in Table 1 as making up the single "yolk zone." Under the heading "Other Granules" are included those granules that were distinctly separated from the yolk spheres or were of an obviously different type. The fifth layer of the *Arbacia* egg (constituting 5 per cent of the egg volume) is centripetal to the colorless yolk layer at the border of the hyaline zone (E. B. Harvey, 1932), and the erythrophilous granules are the heavy red-pigment granules (constituting about 10 per cent of the egg volume) containing echinochrome. In *Urechis* the cerise heme granules are the heaviest substance of the egg (Taylor, 1931). In the egg of *Cumingia*, the pink pigment granules are located centrifugally to the colorless yolk granules of smaller size (Costello, 1934). In these cases the additional zones of granules are included in Table 1 as "Other Granules."

In the egg of *Styela*, as shown in Plate 1, Figure 16, there is an indistinct centrifugally located clear zone. According to Conklin (1931), this is composed of test cells that have not yet been extruded. They have been listed as 6 per cent other granules in the calculations included in Table 1.

Certain centrifuged eggs, showing five or six zones of stratification instead of the customary three, are of particular interest since they possibly indicate that there are two types of hyaline protoplasm of different density. *Nereis* eggs centrifuged with forces of 240,000 times gravity give a very satisfactory picture of these two hyaline zones. The toughness of the egg membrane is advantageous in enabling the *Nereis* egg to withstand the forces necessary to bring about this separation of substances. In certain other cases, lower forces have accomplished a similar separation of materials. Clement (1935, p. 411) has figured an *Ilyanassa* egg showing five zones of separated material, including two hyaline zones. The middle zone, however, is believed by Clement to consist of the mitochondria. In *Urechis*, Taylor (1931) also observed five zones in eggs centrifuged for some hours at a force of 4,800 times gravity. These zones were as follows: the black cap, brown stratum, yellow stratum, hyaline zone, zone of red granules. In my experiments the black cap (oil?) occupied about 7 per cent, the hyaline zone about 49 per cent, the germinal vesicle 9 per cent, and the heme granules about 1 per cent of the egg volume. The remainder of the egg (34 per cent) includes the other granules. Because of the unusual position of the hyaline zone and the absence of a definite zone at the centrifugal pole comparable to the yolk zone of other eggs, it does not seem possible to homologize the zonation of the *Urechis* egg with the zonations of other egg species. For this reason the calculations for the *Urechis* egg have been omitted from Table 1.

The relationship between the substances in the various eggs that are loosely termed "yolk" is unknown. Yolk granules in the different eggs vary in size, color, density, and

staining reactions. For the purposes of this paper, however, yolk may be defined as a substance of density greater than that of hyaline zone I, and not brilliantly pigmented. This definition includes all materials listed as yolk in Table 1, and excludes the pigment granules.

Homologies between the lipoid (oil or fat) layers of the several egg species may be more readily ascertained, since tests for lipoid substances are possible. The oil stratum is the layer of lowest density and therefore occupies the most centripetal position. It is recognized that all the fatty or lipoid substances of the egg are not present in the protoplasm as discrete formed droplets, and that the volume of the oil cap does not represent the total lipoid content of the egg.

The photomicrographs of typical centrifuged eggs (Pl. I, Figs 1-16) show a fundamental similarity of stratification. The classification of the strata into the several categories indicated in Table 1 is therefore considered justifiable. A photomicrograph of a centrifuged *Arbacia* egg showing more satisfactory stratification than that of Plate I, Figure 11, is presented by Heilbrunn (1937, Fig. 14). Costello also figures a number of centrifuged *Cumingia* eggs (1934) and of centrifuged *Asterias* eggs (1935). In Plate I the degree of stratification shown is in some cases submaximal. Other figures, showing greater stratification, but less satisfactorily reproducible, were employed for the calculations.

DISCUSSION

The results included in this paper may be compared with those of other investigators in a few instances. Conklin (1916) observed three zones in the centrifuged egg of *Crepidula plana*. These were the oil zone, hyaline zone, and yolk zone, occupying 2 per cent, 22 per cent, and 76 per cent, respectively, of the egg volume. Considering the difference in magnitude of the centrifugal forces employed (Conklin used a centrifugal force of 2,000 times gravity), these values agree fairly well with the volumes of comparable zones in the gastropod eggs studied (i.e., the nudibranchs *Anisodoris*, *Archidoris*, *Cadlina*, *Diaulula*, *Hopkinsia*, and *Rostanga*). The results in Table 1 may be compared with the measurements of E. N. Harvey (1932) and of Holter (1936), in which corrections were made for the volume of hyaline protoplasm between the packed granules. Allowing for this correction, one may conclude that the results obtained by different investigators upon different lots of eggs within the same egg species may agree within about 10 per cent.

In every case (but one) in which comparisons are possible, the volume of the formed components exceeds the volume of the osmotic dead space. For example, while Białasiewicz (1933) found the osmotic dead space of eggs of four species of echinoderms to vary from 19.3 per cent to 36.4 per cent, my calculations of the volume of the dispersed phase for four other species of echinoderm eggs are as follows: *Arbacia*, 55 per cent; *Asterias*, 80 per cent; *Echinarachnius*, 80 per cent; and *Strongylocentrotus purpuratus*, 72 per cent. The application of the maximal correction for the volume of hyaline protoplasm located between the packed granules reduces these figures to 40.7 per cent, 59.2 per cent, 59.2 per cent, and 53.3 per cent, respectively. McCutcheon, Lucké, and Hartline's value (1931) for the osmotically inert material of the *Arbacia* egg (7-14 per cent) is far below the value for the relative volume of the formed components.

Fauré-Fremiet (1924) studied the osmotic properties of the eggs of *Sabellaria alveolata*. Making certain assumptions based upon the water content of the egg, he esti-

mated that 30 per cent of the cell volume consists of "dry substance." The term "dry substance," originally used by Overton, is, according to Lucké and McCutcheon (1932), equivalent to that fraction of the cell volume which does not take part in osmotic exchange of water.

Lucké and McCutcheon (1932) calculated from the data of Ephrussi and Neukomm (1927) that 46 per cent of the cell volume of the unfertilized egg of *Paracentrotus lividus* is occupied by osmotically inactive material. This is the highest figure recorded for a marine egg, and is of the order of magnitude of the volume occupied by the formed components of related egg species.

The "nonsolvent volume" of the eggs of *Echinometra lucunter* was calculated by Leitch (1937). He found that the nonsolvent volume of the eggs remains relatively constant at an average value of 34 per cent of the initial egg volume for almost 2 hours after spawning. On longer standing before use, the value was increased to 53 per cent.

SUMMARY

The relative volumes occupied by the formed components of centrifuged eggs have been calculated for twenty-six egg species. If these relative volumes are not corrected for the volume of hyaloplasm occupying the interstices between the granules, the volume occupied by the formed constituents varies from 40 per cent in the egg of *Hydroides* to 82 per cent in the egg of *Polychoerus*.

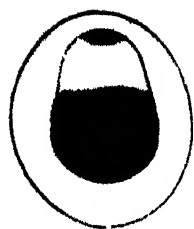
The relationship between the relative quantities of formed components in marine egg cells and certain problems of cellular physiology is discussed.

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PLATE I



6



7



8



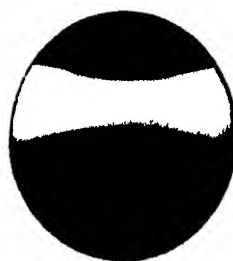
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16

PLATE I

All figures are photomicrographs of living centrifuged eggs. The centripetal pole is uppermost in each case.

FIG. 1.—*Diaulula* egg centrifuged 50 minutes at about $4,800 \times g$., at the time of formation of the first polar body; photographed 9 minutes later. The first polar body is represented by the blurred spot in the hyaline segment.

FIG. 2.—*Hopkinsia* egg centrifuged 8 minutes at about $25,000 \times g$., photographed 6 minutes later.

FIG. 3.—*Rostanga* egg centrifuged 8 minutes at about $26,000 \times g$., photographed 5 minutes later.

FIG. 4.—*Anisodoris* egg centrifuged 7 minutes at about $27,000 \times g$., photographed 8 minutes later. Dark-field illumination.

FIG. 5.—Unfertilized *Asterias* egg centrifuged 12 minutes at $6,000 \times g$., 10 minutes after entering sea water; photographed 2 minutes later.

FIG. 6.—*Archidoris* egg centrifuged 17 minutes at about $28,000 \times g$., before the formation of the first polar body, photographed 31 minutes after centrifuging. Note polar body at the hyaline segment.

FIG. 7.—Unfertilized *Cumingia* egg centrifuged 10 minutes at $6,000 \times g$., photographed 9 minutes later.

FIG. 8.—Unfertilized *Nereis* egg centrifuged 60 minutes at $6,000 \times g$., photographed 8 minutes later.

FIG. 9.—Fertilized *Nereis* egg centrifuged 7 minutes at $6,000 \times g$., 27 minutes after insemination; photographed 8 minutes after centrifuging.

FIG. 10.—Unfertilized *Urechis* egg centrifuged 55 minutes at $4,800 \times g$., photographed 12 minutes later.

FIG. 11.—Unfertilized *Arbacia* egg centrifuged 20 minutes at $6,000 \times g$., photographed 3 minutes later.

FIG. 12.—Unfertilized *Phascolosoma* egg centrifuged 23 minutes at $6,000 \times g$., photographed 15 minutes after centrifuging.

FIG. 13.—Unfertilized *Sabellaria* egg centrifuged 19 minutes at $6,000 \times g$., photographed 19 minutes later.

FIG. 14.—Unfertilized *Echinarachnius* egg centrifuged 24 minutes at $6,000 \times g$., photographed 5 minutes later.

FIG. 15.—Unfertilized *Amphitrite* egg centrifuged 17 minutes at $6,000 \times g$., photographed 9 minutes later.

FIG. 16.—Unfertilized *Styela* egg centrifuged 10 minutes at $6,000 \times g$., photographed 14 minutes later. Note oil droplets caught below membrane of the germinal vesicle.

THE CHEMICAL NATURE OF THE AMPHIBIAN ORGANIZER: III. STIMULATION OF THE PRESUMPTIVE EPIDERMIS OF AMBYSTOMA BY MEANS OF CELL EXTRACTS AND CHEMICAL SUBSTANCES¹

(One plate)

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STUDIES on the chemical nature of the amphibian organizer have become complicated by the fact that so many unrelated substances and extracts will stimulate the formation of a neural plate or neural tube. Fischer and his co-workers (1935) suggest that some property of these seemingly unrelated substances, such as their acidity, is the stimulus for neural tube formation. Waddington *et al.* (1936) have attempted to explain most neural inductions as caused by a sterol present in the substances used, or by a liberation of this sterol within the presumptive epidermis by application of the substances.

TABLE 1
NEUTRAL INDUCTION IN *Ambystoma opacum* BY MEANS OF CEPHALIN
Kaolin used as a carrier and cholesterol in kaolin as control

Substance	Preparation	Operated	Survived	Growth	Neural Plate	Neural Tube
Cephalin.....	A	26	20	7	5	5
Cholesterol.....	B	20	14	1	0	0

The purpose of these researches was to test out lipid extracts for neural inductor capacity and to try to substitute artificial inductors. When it was found that substances like digitonin, acids, and bases were able to act as artificial neural inductors, it was evident that a reinvestigation of the naturally occurring inductor must be undertaken in light of these new facts. For it then becomes important in using any tissue extract to distinguish between an artificial inductor and the natural inductor in the roof of the archenteron. Therefore, a comparison was made of the inductions obtained by lipid extracts and those obtained from protein residues to see where the greatest potency resided.

In an earlier paper (Barth, 1934) the crude cephalin fraction of mammalian brain was used for neural induction in *Ambystoma*. Later (Barth, 1935) it was found that purified cephalin would induce large neural tubes in *Ambystoma opacum*. In the following experiments cephalin was prepared from calves' brain, according to the methods of Parnas and Renall as outlined in Thierfelder and Klenk (1930). The cephalin obtained was mixed with kaolin to form a paste which could be cut into small pieces and placed in the blastocoel with a pair of sharp forceps. As a control, cholesterol prepared from the brain was mixed with kaolin and implanted in the same way.

¹ These researches were aided by a grant from the Rockefeller Foundation for research in chemical embryology.

Table 1 records the results. Fifty per cent of the embryos which survived showed neural induction, and an example is shown in Plate I, Figure 1, where a large irregular tube-resembling brain is induced in the ventral body region. Posterior to the section shown in Figure 1, vesicles are present which are interpreted as otic vesicles. There are no connections between the primary neural tube of the host and the induced structures.

NATURE OF THE ACTION OF CEPHALIN

It is important to know what property of cephalin is responsible for the neural induction, and the next series of experiments were performed to throw some light on its action. Needham, Waddington, and Needham (1934) reported that a substance precipitated by digitonin caused neural induction in *Triton*, and Fischer, Wehmeier, Lehman, Jühling, and Hultzsck (1935) showed that various substances, regardless of their chemical nature, induced neural plates when rendered acid by shaking with acids.

TABLE 2

THE ACTION OF VARIOUS PREPARATIONS OF CEPHALIN, THE PRODUCTS OF HYDROLYSIS OF CEPHALIN, AND STEROL FROM BRAIN ON THE PRESUMPTIVE EPIDERMIS OF *Ambystoma opacum* IMPLANTED INTO BLASTOCOEL

Preparation	Concentration (Per Cent)	Operated	Survived	Growth	Neural Plate	Neural Tube
C (hydrolyzed cephalin)	25 0	30	11		1	2
D (see text)		70	32	3	4	2
E (sterol)	100 0	44	32	0	1	0
F (cephalin)	9 0	16	12	5	1	0
G (cephalin)	3 0	16	13		1	0
H (cephalin)	14 0	22	14		0	0
I (cephalin)	10 0	91	34	32	0	1
J (cephalin)	5 0	24	16	0	0	0
K (cephalin)	0 5	74	12	0	0	0
L (cephalin)		24	13	0	0	0

Accordingly, cephalin was hydrolyzed, and the products of hydrolysis tested and found to be active (Table 2, preparation C, and Fig. 2). Next a sample of cephalin was saponified with 2 N alcoholic potassium hydroxide. After acidification and extraction with ether, the products of hydrolysis were dissolved in warm 95 per cent alcohol and 1 per cent digitonin in 95 per cent alcohol added. No precipitate formed, but, upon cooling 24 hours at 5°C., the solution became cloudy and was centrifuged. The supernatant liquid was evaporated to dryness, and this residue extracted with petroleum ether. This preparation (D, Table 2), was tested and found to be active. Thus the action of cephalin and the product of hydrolysis of cephalin as a neural inducer are not due to the presence of an impurity in the form of a sterol which precipitates with digitonin such as Needham *et al.* found in the ether extracts from glycogen.

The sterol fraction was tested directly in the following manner. Two calves' brains were extracted with acetone twice, alcohol once, and ether twice, and the united extracts evaporated to remove solvents. A portion was saponified for 5 hours with 2 N alcoholic potassium hydroxide, and, after acidification and extraction with ether, the products of hydrolysis were dissolved in warm 95 per cent alcohol and 1 per cent digitonin in

95 per cent alcohol added, and kept overnight at 5° C. A voluminous precipitate was obtained, and the filtrate tested with digitonin until no more precipitate formed.

The sterols were recovered from their digitonides by means of anhydrous pyridine followed by precipitation with petroleum ether. This preparation (E) was very weak in its action, forming a neural plate but no tubes. It seems likely from this result and the foregoing experiments with preparation D that the action of cephalin from the brain is not due to a sterol impurity as suggested by Waddington, Needham, Nowinski, Lemberg, and Cohen (1936).

Since Fischer *et al.* (1935) found that simple acidification of substances caused them to become neural inductors, this possibility was tested in the case of cephalin. Using the method of extraction as outlined in Thierfelder and Klenk (1930), one of the steps is to precipitate the cephalin with HCl, thus forming an acid. The cephalin is then washed with acetone and dried *in vacuo*. Subsequent treatment merely involves precipitation with acetone from ether solutions. The cephalin thus prepared is an acid, and its action as a neural inductor may be due to the acid stimulus of Fischer *et al.*

Several observations indicated that cephalin (preparation A) was toxic. In the first place, after it was inserted in the blastocoel, the cells with which it made contact began to cytolize, and this action sometimes spread until the whole embryo cytolized. Second, in some experiments the preparation was placed on the surface of the early gastrula in contact with the vitelline membrane. Something diffused through the membrane and killed the cells underneath. This action could be observed directly under the binocular microscope. The dead cells formed a layer which lifted off from the living cells. The living cells underneath this dead layer became pigmented and folded, suggesting neural induction resulting from the dead layer. This has not yet been confirmed in sections of the embryos. Finally, the preparation proved to be acid to litmus.

In addition to the foregoing observations, further experiments with different preparations of cephalin in varying concentration gave variable results, some preparations giving induction, others not (see Table 2, preparations F-L). Of special interest in this table is preparation I, which is cephalin which was kept under acetone for a year. Its action is remarkably constant, always stimulating large outgrowths with a few elongate nuclei but never neural plates or tubes (Fig. 3).

A similar preparation of cephalin was implanted in *A. mexicanum* (Table 3, preparation I). Here, too, large outgrowths result as in Figure 3. In this form, as in *A. opacum*, the effect of cephalin was variable, one preparation showing induction, another not (Table 3, I, M, X).

Finally, in *A. punctatum* the results are much the same as in the other species (Table 4). Preparation A, which is very effective in *A. opacum*, has not been tried, and most of the other preparations of cephalin failed to induce. Preparation I, however, stimulates large outgrowths which for the most part show little or no neural induction. Other preparations may occasionally form large growths but not so consistently as preparation I.

Here (Table 4) are also included some tests with synthetic cephalin prepared by two students, Mr. Leonard C. Miller and Mr. James S. Missett. Some of the intermediate products were also tried, such as α - and β -distearin and the di-stearchlorhydrins from these. The latter induced a small neural tube.

Other fractions of calves' brain proved ineffective. Crude lecithin (preparation Q) and crude sterol fraction (preparation R) were negative. Preparation E, the sterol recovered after precipitation by digitonin, also gave no action. This lack of induction in *A. puncta-*

tum with various lipid extracts led to the use of the living dorsal lip of the blastopore as an inductor in order to see whether the epidermis would react under the conditions of the experiment. In these experiments the living dorsal lip was wrapped up in jackets of presumptive epidermis, and good neural induction was obtained. In a series where the dorsal lip was killed with alcohol or dried at room temperature no induction was found, and the dead implants caused cytolysis of the presumptive epidermis in which they were placed. It seems quite clear that the epidermis of *A. punctatum* is not easily stimulated by dead implants or extracts but that it will react to the living organizer. It should form, therefore, a good test object for the naturally occurring organizer.

TABLE 3

EFFECT OF CEPHALIN IMPLANTED INTO THE BLASTOCOEL OF *Ambystoma mexicanum*

Preparation	Concentration	Operated	Survived	Growth	Neural Plate	Neural Tube
I.....	5 per cent in agar	22	21	16	0	0
M.....	100 per cent	23	13	2	0
X.....	100 per cent	33	31	0	0

TABLE 4

EFFECT OF CEPHALIN AND OTHER EXTRACTS OF BRAIN IMPLANTED INTO BLASTOCOEL OF *Ambystoma punctatum*

Preparation	Concentration	Operated	Survived	Growth	Neural Plate	Neural Tube
(cephalin).....	5.0 per cent in agar	38	30	18	1	0
(cephalin).....	0.5 per cent in agar	90	31	6	0	0
(C).....	0.5 per cent in lard	15	9	6	0	0
(synthetic cephalin).....	5.0 per cent in agar	30	17	0	0
(distearin).....	5.0 per cent in agar	15	12	0	0
(distearylchlor-hydrin).....	5.0 per cent in agar	45	18	0	1
(crude lecithin).....	5.0 per cent in agar	26	24	0	0
(crude sterol).....	5.0 per cent in agar	23	12	0	0
(sterol).....	2.5 per cent in agar	25	18	0	0
(cephalin).....	50.0 per cent in kaolin	60	45	0	0

A number of extracts were tried in a large number of cases in one or more of the three foregoing species of *Ambystoma* with no signs of induction. These are sphingomyelin, crystalline preparations of the cerebroside, glycerophosphoric acid, and lecithin.

THE ACTION OF TOXIC AGENTS ON THE PRESUMPTIVE EPIDERMIS

The results with cephalin suggested that it was acting as a neural inductor by virtue of its toxicity and probably its acidity. Only in high concentrations did cephalin act, and even then some samples did not induce. Since the entire ectoderm of the gastrula possesses the ability to induce after being killed in various ways (Holtfreter, 1934), it seemed possible that cephalin, by killing or injuring some of the cells with which it came in contact, caused these killed or injured cells to act as inductors.

Another and more likely possibility is that the formation of the neural tube is a reaction to a stimulus much as an egg reacts to parthenogenetic agents. Many toxic agents, if applied for a short time only, will activate an egg without killing. The action of cephalin, then, may well be a direct stimulus to the cells of the presumptive epidermis, which respond by forming a neural tube. It is not necessary to assume, as have Waddington *et al.* (1936), that foreign substances such as methylene blue act by release of the neural inductor. These substances may act directly by substituting for the normal stimulus of the organizer. There is no evidence that the neural inductor is present in the presumptive epidermis, since all that the experiments show is that, after killing, the dead cells will stimulate a neural tube. It is significant that any source of dead cells will act (Holtfreter, 1934). Since dead cells liberate all sorts of toxic substances which might act as stimulating agents, just as methylene blue and cephalin, and, indeed, the sterol suggested by Needham *et al.* (1934; Waddington *et al.*, 1936) as the naturally occurring inductor, there is as yet no evidence that these dead cells contain the naturally occurring inductor.

TABLE 5
EFFECT OF DIGITONIN ON THE PRESUMPTIVE EPIDERMIS OF
Ambystoma mexicanum

Neural induction as in Figs. 4 and 5. Useful cases are those in which the implant is in contact with the epidermis. Concentration is percentage by weight in powdered egg albumen or in lanoline.

Concentration of Digitonin (Per Cent)	Useful Cases	Neural Induc- tion as in Figs. 4 and 5	Percentage
10.0.....	6	4	66
1.0.....	9+2*	5+1	55
0.5.....	15	12	80
0.1.....	10	8	80
0.05.....	7	5	70
Total cases.....	47+2	34+1	70

* Two cases of explants of presumptive epidermis, one of which gave typical reaction.

Certainly, the wide variety of substances and tissues which stimulate the presumptive epidermis to form a neural tube do not suggest that we are dealing with a naturally occurring inductor any more than the wide variety of parthenogenetic agents throw any light on the naturally occurring stimulus in the sperm.

In any event foreign substances applied to the epidermis *do* cause neural tubes to form, and so other substances known to be toxic to cells were tried. Digitonin was used because it was possible that in some of the earlier work of Needham *et al.* (1934) the action of the sterol may have been due to the digitonin which was not separated from it.

Digitonin was therefore mixed in varying concentrations with lanoline and also with powdered egg albumen as a carrier and these mixtures implanted into the blastocoel of *A. mexicanum*. The reaction is remarkably constant when the implant comes in contact with the presumptive epidermis. The cells are converted into a small plate resembling the cells in the neural tube very closely (Figs. 4 and 5). Table 5 records the results with varying concentrations. It is seen that digitonin acts in low concentrations, and the

lowest tried, which was 0.05 per cent, was still active. Similar results were obtained with *A. punctatum* where, out of 18 useful cases, 5 showed the typical digitonin reaction (Figs. 4 and 5).

USE OF OTHER TOXIC AGENTS

The fact that cephalin acted as an inducing agent in toxic concentrations in *A. opacum* and that digitonin caused a similar reaction in *A. mexicanum* and *A. punctatum* suggested that other toxic agents might stimulate. Since Fischer *et al.* had already shown that fatty acids and acid nucleoprotein, etc., would induce, it seemed probable that H-ion concentrations outside the physiological range might also stimulate. Using powdered egg albumen as a carrier, buffers at various pH were mixed with the albumen and inserted into the blastocoel. This method does not give the pH of the egg albumen in contact with the epidermis, and the pH indicated in this paper is merely that of the original phosphate or borate buffer before mixing with the egg albumen.

TABLE 6

THE ACTION OF VARIOUS BUFFETS UPON THE PRESUMPTIVE
EPIDERMIS OF *Ambystoma mexicanum* AND *A. punctatum*

Reaction as in Figs. 4 and 5. Useful cases are those in which the implant is in contact with the epidermis. Phosphate or borate buffers mixed with powdered egg albumen as a carrier.

pH of Buffer	Total Cases	Useful Cases	Neural Induction
3.0	36	12	7
4.0	21	10	0
5.0	15	6	1
Egg albumen control	18	10	0
8.1	17	6	1
8.7	13	11	0
9.7	39	22	7

Table 6 shows that the most effective buffers are those at pH 3.0 and 9.7—the extremes of the range tested. The numbers are admittedly small, but the main point here is that both acid and alkaline buffers stimulate. In comparing the effect of buffers with digitonin, it should be pointed out that the reaction with digitonin is more constant and a little more extensive.

THE QUESTION OF THE NATURALLY OCCURRING INDUCTORS

In view of the results presented in this paper and those of Fischer *et al.*, considerable doubt as to the lipid nature of the neural inductor is aroused. If foreign substances introduced into the blastocoel produce changes in the cells similar to those produced by the neural inductor of the organizer, it is extremely difficult to know when one is working with the natural occurring inductor. Careful quantitative and qualitative studies on the response of the ectoderm to the chemical stimulus in various concentrations will help; but, finally, the substance claimed as the naturally occurring inductor must be shown to be present in the roof of the archenteron.

Certainly, if the inductions described in this paper and also in the papers of Needham *et al.* and Waddington *et al.* (1936) are compared with those obtained by the natural or-

ganizer, one is immediately struck by the low percentage of induction and the low degree of differentiation of the chemically induced neural tubes. The lack of mesodermal structures and sensory structures is conspicuous. Further, a comparison of Holtfreter's inductions with protein residues after ether and alcohol extraction with Needham's inductions with sterol shows clearly that the greater potency is in the protein residue. Both of these investigators used the same test object, *T. taeniatum*.

Accordingly, a series of experiments with fat-free calves' brain and frogs' gastrula have been started in order to compare induction with various lipids. The fat-free fraction of calves' brain was prepared by drying in two changes of acetone, followed by extraction with alcohol and two extractions with ethyl ether. A sample of the finely divided residue was then treated with boiling 95 per cent alcohol for 11 hours and filtered hot. It was further treated with boiling ether filtered and refluxed once more with 95 per cent alcohol for 3 hours, filtered, and the process repeated. The residue was finally boiled with ether and filtered.

This residue gave very good induction of neural tubes in 80 per cent of the cases when implanted in *A. opacum* (Fig. 6). In ten cases where the implant came in contact with the presumptive epidermis, eight showed good neural induction. There may possibly be some either soluble substances left in this residue, and so this possibility was tested in the following way.

After preliminary extraction of the brain with acetone, alcohol, and ether some of the residue was treated with pepsin HCl for several days, after which the mixture was extracted with ethyl ether. The ether was removed, and the lipid picked up with petrol ether. This lipid extract was tested in 10 per cent, 1 per cent, and 0.1 per cent concentration in egg albumen in *A. mexicanum*. In a few cases a reaction of the epidermis was obtained which was identical with that produced by digitonin and acid buffers at pH 3. No neural tubes were obtained. This lipid is not the active principle of the fat-free residue. However, there may still be protein lipid or glycogen lipid combination not broken up by pepsin-HCl or extracted by prolonged fat solvents, and this possibility must be tested by other methods of hydrolysis of the proteins.

DISCUSSION

Recent reviews (Weiss, 1935; Woerdeman, 1936) have pointed out the difference in the action of the living organizer and the action of dead organizer and chemical substances. In the first place, living organizer loses its specificity for inducing particular structures when killed by various means (Holtfreter, 1934). All parts of the organizer after killing behave like any source of dead tissue. Second, when attempts are made to extract active principles, they give only neural tubes, and the associated structures like the sensory structures are lacking. Finally, when relatively pure substances are used, only small plates or tubes are elicited. In each of these steps from living organizer to pure substances something is lost, and a satisfactory chemical preparation to be used as organizer is still wanting. From a comparison of protein residues and lipid extracts, it appears that most of the potency remains in the residue.

It is suggested therefore that the protein residue may contain the naturally occurring organizer and that the lipid extract merely acts as does digitonin, di-benzanthracene, and other artificial stimulators. It is highly significant in this respect that all Holtfreter's treatments of the organizer—heat, drying, HCl, etc.—lead to a denaturation of the proteins with the result that the specificity of induction is lost.

Induction of neural structures is, then, the result of a general stimulus applied to the presumptive epidermis. The inductors in the organization center, however, have a specific effect which has been lost in all attempts to extract the substances responsible for this specific action. The reactivity of the presumptive epidermis includes formation of all the sensory structures, notochord, mesodermal somites, pronephros, and other structures. None of these structures has as yet appeared as a result of chemical stimulation, and only rarely have they appeared with dead organizer.

The fact that digitonin, acids, bases, and other substances producing cytolysis act as neural inductors recalls the case of artificial parthenogenesis in the sea urchin, where similar agents stimulate development. In both cases the stimulus is of a general nature acting on a preformed mechanism which is called into action by a variety of substances.

There are apparently two methods by which a neural tube may be stimulated to form from presumptive epidermis: first, by a variety of dead tissues and chemical agents which may be classified as artificial inductors and, second, by some specific substance or substances in the organizer which are natural inductors. The nature of these substances is not known, but my experiments suggest that they are proteins.

SUMMARY

1. Cephalin acts as an inductor of neural tubes in *A. opacum* only when it is somewhat toxic because of its acid properties.
2. Other toxic agents such as digitonin, acid, and alkaline buffers will stimulate the formation of a neural plate in *A. mexicanum* and in *A. punctatum*.
3. A comparison of protein residues and lipid extract shows the potency for neural induction to be greater in the protein residue.
4. The suggestion is made that substances previously used as inductors are artificial inductors and that the naturally occurring inductors are probably proteins.

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PLATE I

Induction of neural structures in the epidermis of *Ambystoma*. All figures show the induced neural plate or tube in the ventral body region, and the induced structures have no connection with the host neural tube. The implant in all cases is adjacent to the induced structure.

FIG. 1.—Induction of neural tube in *Ambystoma opacum* by means of cephalin. Preparation M used.

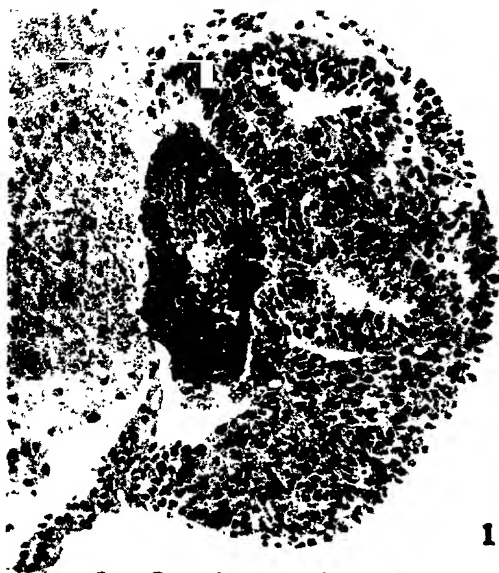
FIG. 2.—Induction of neural plate and folds in *A. opacum* by means of the products of hydrolysis of cephalin.

FIG. 3.—Large epidermal proliferations induced by cephalin in *A. opacum*. Preparation I used.

FIGS. 4 and 5.—Inductions of neural plates in *A. mexicanum* by means of digitonin.

FIG. 6.—A neural tube induced in *A. opacum* by the protein residue after extraction of lipids.

PLATE I



5

6

STUDIES ON THE ANTAGONISM BETWEEN IMPLANT AND HOST IN FISH EMBRYOS

(Two plates)

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WHILE studying the action of certain drugs on the innervated and denervated fish heart (Brinley, 1935), it was found desirable to ascertain the effect of certain drugs on a pulsating heart which had been excised from its normal blood supply and nerve connections and implanted into the side of the yolk sac in another individual in such a position that it could be easily observed and would not likely be innervated by nerves from the host. These implants developed and continued beating for a maximum period of 12 days. At the end of that time, the rate and amplitude of the host heart gradually decreased until finally death occurred prior to stoppage of the contractions of the implant. This was a very surprising result, and a search of the literature revealed no exact parallel case. So it was deemed advisable to restudy the problem in detail to see if more light could be thrown on the general problem of the incompatibility between host and implant.

The great majority of the work on organ transplantation has been done on amphibians, with some work on birds and mammals, but little has been reported on fish (Koppanyi, 1923; Blatt, 1924; Mangold, 1931). In the work here reported we have been especially interested in studying the physiological action of different types of implanted tissue on the host, using *Fundulus heteroclitus* as the donor and *F. majalis* or hybrids between the two species as recipients, and homoplastic transplants with brown trout (*Salmo fario*) and the hardhead minnow (*Hepsetia stipes*). Three types of tissue were selected for the experiment: (1) the heart, as representing a large, well-organized and actively functioning structure; (2) the eye, also large and well organized, but passive, with activity apparently limited to growth changes; and (3) glandular tissues, the liver and spleen, with the thought that they would present an intermediate type of activity, not being as well organized as the others and falling somewhere between them in functional activity.

HEART

In all the experiments reported on the heart unless otherwise stated *F. heteroclitus* was used as the donor and *F. majalis* as the recipient. The embryonic heart develops early and begins to pulsate at room temperature on the third to fourth day after fertilization. It is relatively large and stands out in plain view, upon the anterior surface of the yolk sac. It beats forcibly and regularly at a uniform rate of about 130-40 beats per minute at temperatures of 20°-22° C.

* The writer wishes to express his appreciation to the Carnegie Institute of Washington and to the director of the Dry Tortugas Laboratory, Dr. D. H. Tennent, for the opportunity of conducting a portion of the work at that laboratory.

In preparing the heart for implantation, *F. heteroclitus* embryos from 5-10 days after fertilization were placed in 60 per cent sea water (60 parts sea water, 40 parts distilled water), which is nearly isotonic with the excised heart. The chorions were removed with fine dissecting scissors, and the naked embryos floated out in the dilute sea water. This operation does not injure the embryos, as is evidenced by their ability to develop normally. The heart, which consists of a single auricle and ventricle, can be seen in its normal position and is covered by a thin membrane, the pericardium, which seems to form a part of, or unites with, the membranes of the yolk sac. After removal of the chorion, the pericardium is cut with a fine needle scalpel and carefully pulled away from the anterior portion of the yolk sac, leaving the normally beating heart exposed but still connected to the host blood vessels. The aorta is then cut where it leaves the heart, and, by gently elevating and pulling the tip of the ventricle, the heart can be lifted away from the yolk sac and finally severed by cutting the veins. The excised heart will continue to beat in isotonic sea water for 3 or 4 hours. Implantation may be made at any time during this period, but the best results are obtained if the transfer into the host is made as soon as possible after the heart is taken from the donor. In all cases here reported the entire heart (auricle and ventricle with the cut ends of the arteries and veins) was used as the implant; but, if the heart is divided at the auriculo-ventricular junction, either portion will continue to beat when implanted into the host. The auricle will soon resume contractions at the normal rate, but the isolated ventricle always beats much slower, often only a few beats per minute.

In preparing the embryos of *F. majalis* to receive the implant, the chorions are removed from embryos 8-10 days after fertilization, at least a day before implantation, and only embryos are used which are in good condition with strong, well-established circulations. The naked embryos are then placed in isotonic sea water, and a very small slit is made through the membranes where the yolk sac joins the embryo. The cut is made just back of the large vessels which carry blood to the yolk.

The pulsating heart, previously prepared, is quickly transferred with teasing needles to the slit made in the yolk sac and held in place for a few seconds until the membranes close around the implant and hold it in place. The implant must not be pushed into the yolk so that it will be submerged when the cut in the membrane is repaired. For a more detailed report of the operative technique the reader is referred to a previous paper (Brinley, 1935).

In the majority of cases the implant did not stop beating while it was being removed from the donor and transplanted into the host. The rate of contraction slowed considerably for the first few hours after the operation but gradually increased until the fourth or fifth day, when the normal rate was reached and maintained. The implant pulsates as rapidly as the host heart but does not synchronize with it. Usually both chambers of the heart pulsate at a one to one ratio, but at other times only the auricle contracts; or both chambers may contract independently of each other. The inclosed blood corpuscles, carried over in the implant at the time of operation, can be seen to ebb and flow as the heart contracts. In only a few cases did the host blood vessels make contact with the implant. In one case a host capillary grew into the walls of the implant, and in one other case a host capillary entered and carried blood into the lumen of the implant, and the blood was forced back into the capillary at each contraction of the heart. In numerous cases host capillaries developed in the membranes surrounding the implant.

The average length of life of the implant and host was 5-6 days, but some of them lived a maximum of 12 days. The initial effect of the implant on the host was found to consist of a slowing of the ventricular beat which rapidly passed to complete stoppage. Slight contractions of the auricle continued for several minutes, and finally the entire heart stopped in diastole within 3-4 hours after the first effect was noticed. In practically all cases the death of the host preceded by a few hours the death of the implant. In these cases the beating of the implant could be observed, after the heart and circulation of the host had stopped. This reaction was seen when both *F. majalis* and the hybrids were used as hosts. If the implant did not "take," the shriveled remains appeared as a white coagulum on the surface of the yolk sac and did not interfere with the normal activity of the host. Therefore, death of the host is evidently due not to the presence of foreign tissue of protein but probably to the added amount of waste material or to some hormone or humor produced by the actively beating implant which is antagonistic to the heart of the host.

Histological studies of sectioned material show that the implant developed between the ectoderm and mesoderm or between the mesoderm and entoderm. The ectoderm adjacent to the implant shows considerable thickening, which appears to be due to a healing-over and a piling-up of cells and not to multiplication, as only a few of these cells were observed undergoing division. Numerous host capillaries could be seen in the mesoderm covering the implant. In several experiments the host membranes did not grow over the implant, and in these cases the implant grew better than when placed under the ectoderm. The cardiac cells grew out over the adjacent ectoderm and formed large globular cells. In the living condition these cells appeared as minute bunches of grapes which sloughed off or could be picked off with dissecting needles (see Brinley, 1935). The implant retains its identity, and the cells are large with prominent nuclei. The ventricle remains as a thick-walled, compact mass of cells. The auricle tends to spread out in a thin sheet of loosely connected cells. Mitotic figures were observed in both myocardial and endocardial cells (Pl. I, Fig. 1), which indicates that there is considerable cell multiplication and growth of the implant. On the whole, there was surprisingly little change in the histological picture of either the cardiac cells or the contained blood cells. These implanted hearts behaved quite uniformly as they would have done in their natural relations, undergoing little change even after 10 or 12 days. The interesting development in this work was the death of the host prior to stoppage of the contractions of the implant. This point will be discussed later.

The ectoderm overlying the implant always shows marked changes. There is some proliferation, but the most striking occurrence was the change in shape of the surface cells, which become elongated and club-shaped with vesicular nuclei in the distal ends. No other host tissue changes were noted.

EYE

The eye of *F. heteroclitus* differentiates rapidly, and, at the end of the third day, the optic cup is well developed and the lens has formed. Within 5 or 6 days pigment develops in the retina and choroid, and the eye gradually becomes darker and glassy in appearance. Heteroplastic implants were made between *F. heteroclitus*, as the donor, and *F. majalis*, the recipient. The chorions were removed and embryos prepared for operation as described in the previous section. The entire eyeball was carefully removed from the donor, at any time after the third day, so as not to puncture the mem-

branes and allow the humor to escape. In the first series of experiments the entire eye was placed in a small slit made in the pericardium of the host anterior to the yolk sac. This can easily be done without injury to the host or without danger of puncturing the eye with resulting loss of humor. The implant was oriented so that the lens faced outward. The implant developed normally, and pigment appeared in the retina and choroid at the same time as in the controls. The implant was pulled ventrally by the pericardium as the yolk of the host was consumed until finally the implant takes a position adjacent to, and in front of, the heart. In some cases the pericardium did not completely surround the implant, and the eye could clearly be observed with the lens projecting from the membrane. The implant at this stage appears quite normal but considerably smaller than the eyes of the controls.

A histological study of the implanted eyes shows no decided change in the retina or lens; the optic nerve regenerated and grew from the eye into any adjacent tissue, such as the myocardium, liver, striated muscle (Figs. 2, 3, 4), or, in one case where the implant was placed next to the normal eye of the host, the regenerated optic nerve followed the contour of the normal eyeball and entered the brain.

As the implant grew and developed in the pericardial cavity, it often crowded the liver or heart out of place, but there seemed to be no other effect upon the host. The only effect noted on the implant was some distortion due to pressure exerted by the surrounding tissues. In some individuals the pericardium grew over and completely surrounded the implant, and in these experiments the eye was gradually absorbed as indicated by a reduction in the size of the eyeball, disappearance of the optic nerve and lens, and, finally, after considerable time, by a complete disappearance of the pigmented layers of the retina and choroid.

Owing to the difficulty of observing the implant in the ventral position, when the host begins to swim, and absorption of the eye when covered by the pericardium, a second series of experiments was conducted by implanting the eye in the yolk sac, in the same location as the implanted heart (i.e., upon the yolk sac adjacent to the embryo). In this position the implant frequently developed between the mesoderm and ectoderm, many capillaries entered the eyeball, and the implant became pigmented and developed normally (Fig. 5). There seemed to be no effect on the host, and no absorption of the implant had occurred when fixed at the end of a month.

In order to obtain more data on the growth of the optic nerve and its possible connection with the nervous system as indicated in the foregoing experiments, a third series of experiments was conducted by placing the implanted eye between the two normal eyes of the host, so that the regenerating optic nerve would be in a position to connect with nervous tissue. For these experiments, homoplastic implantations were made on brown trout embryos obtained from a Minnesota state fish hatchery and kept in finger bowls in a refrigerator maintained at 10° C. (Brinley, 1937). When the circulation was well established and the eyes began to show pigmentation, the chorions were removed from the embryos, and an eye of the donor was carefully removed in isotonic salt solution and placed in a slit cut in the developing skull of the recipient dorsal to the forebrain between the two normal eyes (Pl. II, Fig. 6). In order to facilitate the operation and to keep the host quiet until the implant was firmly established, it was necessary to anesthetize the host. This was done by placing it in a saturated solution of chloroform. When body movements ceased, the embryo was transferred to a small dissecting tray constructed by partly filling a Syracuse watch glass with melted paraffin and cutting

a small circular hole in the paraffin, sufficiently large to receive the yolk sac of the embryo. The head and tail of the embryo were allowed to rest in a groove in the paraffin. A longitudinal incision was made with fine dissecting needles through the skull at the base of the forebrain and the implant inserted. The embryo was then returned to the refrigerator, and, by the time it recovered from the influence of the anesthetic, the incision was sufficiently well healed to hold the implant in place so that it was not dislodged by swimming movements of the host. Many of these implants "took well" and developed normally until the hosts were fixed for sectioning at the end of 30-120 days. The implant always remained smaller than the normal eye, capillary circulation was established, but no movement of the implant was noticed. Histological study of the implant was made after fixation in Bouin's solution and staining with Delafield's haematoxylin. A rather superficial examination indicated normal development. A layer of ectoderm of the host grew over the implant, the lens and retina seemed normally developed, but no comparative cell counts were made. The optic nerve regenerated from the implant and grew toward the brain; usually it did not actually enter the nervous system but wandered along its surface. In one very interesting case, the optic nerve split into three branches after leaving the eye. One branch grew anteriorly, ending in a mass of loose nerve tissue adjacent to the implanted eye. A second branch followed a capillary blood vessel through the fibrous covering of the brain and apparently ended in the region of the nerve cells of the prosencephalon. The third branch grew caudalward alongside the brain; its end relations could not be determined (Figs. 7, 8). It is doubtful if the second branch could have passed through the fibrous covering of the brain if it had not followed the blood capillary. In another interesting case the path of the nerve was obstructed by cartilage, and the nerve grew around the cartilage and entered the brain (Fig. 9).

The implanted eye seemed to have very little effect upon the host as was evidenced by normal development until the host was fixed for study at the end of 30 days in some *Fundulus* experiments or at the end of 80 days in the case of the trout.

In order to obtain further data on the effect of the implant upon the mortality of the host, a series of experiments was conducted, using glandular tissue as the implant, as it seemed probable that gland tissue would occupy an intermediate position between the very active heart and the as yet nonfunctional, hence passive, eye. The liver and spleen were chosen for these experiments because they develop rather early and can be quite easily removed from the living embryo. These studies were made at the Carnegie Laboratory at Dry Tortugas, and the fish used was the hardhead minnow, *H. stipes* (identified by Dr. S. F. Hildebrand). These minnows spawn during the summer months, from the middle of June to September. The eggs are about a millimeter in diameter and may be obtained either by stripping the female or the adults may be kept in specially constructed "live boxes" containing false bottoms in which cheesecloth trays are placed to receive the eggs when they are naturally spawned and fertilized during the night. Long adhesive threads which are attached to the eggs hold them in the trays. The trays can be transferred to running-water aquariums, and the eggs removed as needed. The eggs hatch in 8-9 days. Owing to some undetermined cause, the mortality of these young fish is very high, even under the most favorable culture conditions, but a sufficient number survived to enable us to make some observations which are included here. The pale-green gall bladder can be distinctly seen in the living embryo on the fourth day and can be used as a landmark in locating the liver. The spleen is identi-

fiable at about the same time as a faintly pink oval body which becomes bright red in a day or two. These glands can be easily removed in isotonic sea water, and the entire gland (exclusive of the gall bladder) can be inserted into the yolk sac of the host. In these experiments the liver or spleen was removed from the host embryo from 6–8 days after fertilization and homoplastic implants made into embryos of the same age. Owing to the high mortality in these embryos, experiments and controls, our experiments were not as successful as we had hoped for. Out of a total of 89 liver implantations only 5 went on to any considerable degree of development. In the majority of the cases where the host survived for a sufficient length of time, the implanted liver was rapidly absorbed, often within 24 hours, usually within 2 days. In one of the cases that survived, the implant was placed alongside the liver of the host, and the two livers were completely fused when fixed after 48 hours (Fig. 10). The blood vessels of host and implanted liver appeared to connect up, and the line of fusion was indistinguishable. All five of these animals were active and apparently normal up to the time of fixation. In another experiment all except a very small portion of the implant was absorbed at the end of 24 hours. In the three remaining cases the implants had become well established between the mesoderm and entoderm of the host, and a few small capillaries entered the implant from a large blood vessel in the mesoderm. A few cells showed mitotic figures, and the implants appeared normal in all respects (Fig. 11).

Ninety-seven spleen implantations were made as already described. At the time of operation the spleens were well formed and bright red, due to the inclosed erythrocytes. The glands were handled carefully to avoid injury and the consequent escape of the blood cells. Owing to the high mortality, only a few of the experimental animals developed sufficiently to be of much value. In nearly all cases the implanted spleen rapidly faded in color so that at the end of 2 or 3 days the implant was not visible in the living host. In three sectioned specimens the implant was plainly visible and filled with erythrocytes at the end of 72 hours. In two cases the implant was very distinct, imbedded in connective tissue anterior to the liver (Fig. 12). These implants were well developed and somewhat smaller than the normal host glands. The hosts were actively swimming and apparently normal at the time of fixation. In one case the implant appeared as a bud on the side of the yolk sac. Histological study showed it to be encapsulated by ectoderm. It was much smaller than the normal spleen, but the inclosed erythrocytes showed no evidences of fragmentation or other abnormal changes.

As far as could be determined in such a short period of time and with only these few cases, there was no apparent effect on the activity or behavior of the host, and histological sections showed no host abnormalities.

CONCLUSIONS AND SUMMARY

The experiments presented here have dealt with 38 successful implantations of the embryonic hearts of *F. heteroclitus* to *F. majalis*. Thirty homoplastic eye implants were made with *F. heteroclitus*; 4 heteroplastic eye implants from *F. heteroclitus* to *F. majalis* and an equal number from *F. heteroclitus* to *F. majalis*-*F. heteroclitus* hybrids. Some 30 homoplastic eye implants were made with brown trout (*S. fario*). In the glandular series 89 implants of the liver and 7 of the spleen were made, using the minnow *Hepsetia*; but, owing to the excessively high mortality in both controls and experiments, only 11 of the experimental animals—5 liver and 6 spleen—lived long enough to present evidence that we considered to be of any value.

In every case the experimental animals were studied daily as a check on the behavior of both host and implanted tissues in the living state and at intervals individuals were killed in Bouin's solution sectioned 10 μ thick and stained with Delafield's haematoxylin and eosin and studied histologically.

A great majority of the heart experiments were successful, the host surviving for a number of days, the implant beating strongly and usually showing mitoses in both myocardial and endocardial elements, and some invasion of the implant by host vessels was observed. The peculiar feature was that the host animal never survived the operation longer than 12 days, and that the host invariably died, i.e., its heart and circulation stopped, before the implanted heart ceased pulsating.

In the case of the eye experiments most of the implants were absorbed when placed in the pericardial cavity of the host, several survived but were much smaller than the eyes of the host at the end of 30 days when fixed for histologic study. When the implants were made in the head region, near the dorsal wall of the forebrain in trout embryos, several lived and developed until killed at the end of 80 days. In no case did we observe any evidence of any physicochemical antagonism between implant and host, and only minor histological changes occurred in certain tissues.

In the few liver and spleen experiments that survived the parasites or other causes, there appeared to be no physiological antagonism between the host and implant or any marked histological changes.

The incompatibility of the implanted heart and host, which resulted in the death of the host, seems to be due to the production of some chemical substance (waste or humor) by the implant which has an inhibitory effect upon the host, resulting in stoppage of the heart. This effect did not occur in the eye experiments or, as far as could be determined, in those of either the liver or the spleen, although in all the liver cases the actual amount of tissue implanted was much greater than in the heart experiments.

It seems from the results presented that an active implant, such as the heart, produces a specific chemical substance which inhibits the function of the same or different organs in the host, while less active organs either do not produce such a substance, or, if produced, it occurs in amounts too small to have any decided effect upon the host.

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PLATE I

FIG. 1.—120 hours after implantation, showing cell undergoing mitosis (*X*) in wall of ventricle. Oil immersion.

FIG. 2.—Regeneration of optic nerve (*ON*) and growth into wall of auricle (*A*). *L*, liver; *V*, ventricle. High power.

FIG. 3.—Growth of regenerated optic nerve (*ON*) into ectoderm (*Ec*). High power.

FIG. 4.—Growth of optic nerve (*ON*) through pericardium (*P*) into liver (*L*). High power.

FIG. 5.—Implant eye (*I*) normally developed in side of embryo posterior to normal eye (*E*). *Y*, yolk. High power.

FIG. 6.—Implant (*I*) in position between normal eyes. Low power.

PLATE II

FIG. 7.—Regeneration of optic nerve (*ON*) and the entrance of one branch (*1*), following blood capillary into brain (*B*). High power.

FIG. 8.—Drawing of foregoing section showing division of regenerated optic nerve (*ON*) into three branches (*1*, *2*, *3*). 39 days after implantation.

FIG. 9.—Drawing of the regenerated optic nerve (*ON*) passing around cartilage (*C*) which obstructed the nerve path. 104 days after implantation.

FIG. 10.—Implanted liver (*L*) fused with host liver (*HL*). Blood capillaries passed from host liver to implant. 72 hours after implantation. High power.

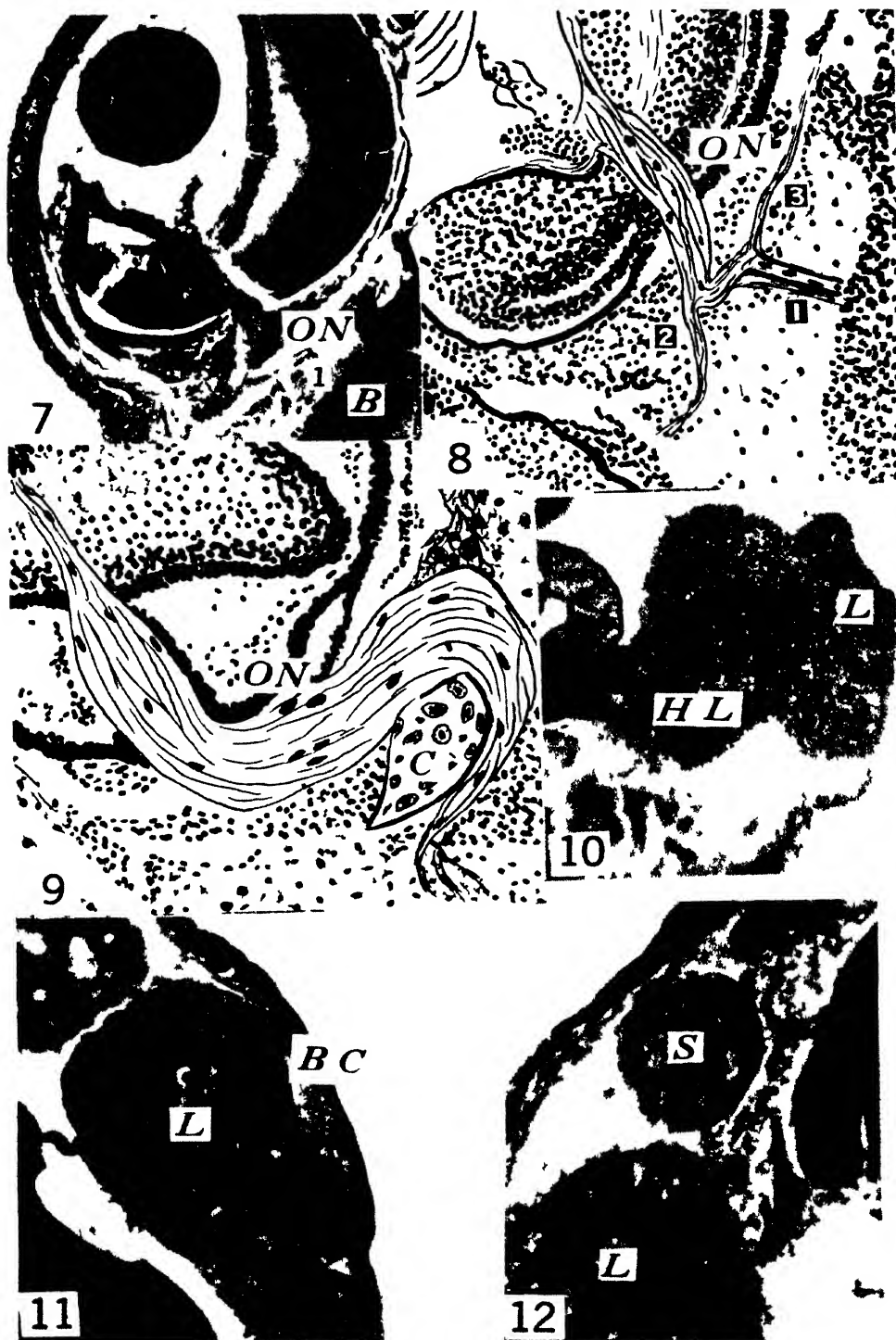
FIG. 11.—Implant liver (*L*) in yolk sac under mesoderm. Blood capillary (*BC*) sending branches (not shown) into implant. 72 hours after implantation.

FIG. 12.—Implant spleen (*S*) imbedded in mesoderm, adjacent to host liver (*L*). High power.

PLATE I



PLATE II



CALCIUM AND POTASSIUM DEFICIENCY AS POSSIBLE CAUSES OF CERTAIN DELAYED FETAL MOVEMENTS

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ANGULO (1930, 1933*a*, 1933*b*, 1934) described spontaneous slow waving movements of the limbs of young rat fetuses occurring some time after ligating the umbilical cord. Presumably, as in other experiments (Angulo, 1932), the specimens were studied in a bath of physiological saline solution. He suggested that ligation of the umbilical vessels had caused "endogenous stimulation" of spinal motor centers by metabolic carbon dioxide, and proposed the theory that, "if the umbilical cord of any fetus is ligated, it will recapitulate the maximum degree of physiological activity it has achieved, but in the inverse order of its normal development."

While observing the same phenomenon in fetal rats, sheep, and cats, it seemed that the movements did not always manifest themselves in the orderly sequence indicated by Angulo's theory, and it appeared questionable that they were activated by the central nervous system. Certainly, they were not the first simple reflex-like movements which can be seen only under good physiological conditions before asphyxia begins (Windle, Orr, and Minear, 1934; Carmichael, 1934; Barcroft, Barron, and Windle, 1936). Therefore, it was decided to examine them in some detail to determine, if possible, their nature.

METHOD

Sixty-nine cat embryos and fetuses between 13.5 and 55 mm. C.R. length and 23-38 days' insemination age were obtained from animals previously decerebrated or under ether anesthesia. The umbilical cords were ligated or cut to allow bleeding; and the specimens were placed separately in small dishes of amniotic fluid (cat), distilled water, Locke's solution, 0.7 per cent sodium chloride, 0.9 per cent sodium chloride, or isotonic (6 per cent) sodium hexametaphosphate. Most of them were allowed to cool to room temperature, but a few were maintained at body temperature. They were watched constantly, usually by several observers, for periods of about 2-4 hours; and timed records of activities were kept.

RESULTS

The delayed spontaneous movements described by Angulo were observed in nearly all of the cat fetuses when the proper solutions were used. They were most commonly seen in the limbs and tail but, at one time or another, involved many other parts. Limbs moved at proximal or distal joints and the plane of movement varied; even the digits could be seen to react. Rotation of the head and flexion of the trunk were encountered less frequently; and movements of the ears, jaw, and abdominal muscles were observed in a few specimens. In the smallest, 13.5-14 mm. long, forelimbs were the only reacting parts. There was no consistent order of appearance of activities, even in fetuses of the same age and size, and thus no absolute relationship to the order of normal development

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of behavior. In some the tail, in others the wrist, elbow, shoulder, or hind-limb joints, were involved in the first movement.

Although isolated movements were often observed, they were usually repeated several or many times in rhythms of activity. Commonly, the tail would be flexed at the rate of once every 4 seconds, while a limb moved simultaneously in rhythm of one beat in 3 seconds.

The movements of the type under consideration were not encountered in fetuses whose placentas were still attached to the uterus with the circulation intact. As reported in a preliminary note (Windle and Orr, 1934), there was a relationship between time of delivering a fetus into the bath of physiological saline and the time of cutting the umbilical cord. The longer a fetus had been exposed to the salt solution before cutting its cord, the sooner the movements appeared afterward. When specimens were removed by severing their umbilical cords and placed in salt solution immediately after opening the uterus, movements began in about half an hour.

All fetuses placed in 0.7 per cent or 0.9 per cent sodium chloride, except one 13.5-mm. specimen, exhibited spontaneous activities. The movements continued long after the fetuses had cooled, their hearts had ceased to beat, and their reflexes could no longer be stimulated with strong faradic shocks. Spontaneous rhythmical movements were observed more than 5 hours after cutting the umbilical cord in one experiment. In distilled water a few strong reactions appeared within 20 minutes but soon stopped. Potassium-free Locke's solution gave results slightly less striking than did the sodium chloride solutions. In calcium-free Locke's, the responses appeared more slowly and were weaker; several fetuses showed no spontaneous movements in calcium-free Locke's solution. Those placed in the solution of sodium hexametaphosphate, which has the property of rendering calcium unusable physiologically (Danforth and Ivy, 1938), reacted very quickly; but movements did not persist long.

In cat's amniotic fluid the waving movements failed to appear. Similarly, Locke's solution brought about activity in no fetus smaller than 53 mm. C.R. length. Three of five 53-55-mm. specimens which had been placed in Locke's did execute weak tail and limb movements. However, three others from the same litter of eight placed in sodium chloride at the same time showed very strong responses of tail, limbs, head, and ears.

Fetuses of various sizes which had failed to move in Locke's solution or amniotic fluid were transferred to the other solutions. There they invariably became active. It took longer for movements to appear after transferring from amniotic fluid to sodium chloride than from Locke's to sodium chloride. Similarly, those which were active in calcium- or potassium-free solutions stopped moving when placed in Locke's. Transfers were repeated several times in a number of experiments, and the fetuses moved or became quiet according to the nature of the solution.

COMMENT

The spontaneous contractions of the developing skeletal muscle after ligating or cutting the umbilical cord were the result of alterations in the surrounding medium. They were not due to accumulation of metabolic carbon dioxide in asphyxia; had they been, they should have appeared as readily in amniotic fluid or Locke's as in other isotonic solutions.

The fact that they occurred in calcium-free Locke's and in sodium hexametaphosphate, which renders calcium inactive physiologically, suggests that a deficiency of cal-

cium ions is one of the causative agents. The importance of proper calcium balance is well established in physiological investigations. That calcium unbalance causes spontaneous skeletal muscle-twitching has been observed by Jasper (1938). Lehmann (1937*a*, 1937*b*) demonstrated spontaneous discharge occurring when the threshold falls with an increase in alkalinity and when it is lowered by exclusion or deionization of calcium. Factors other than calcium deficiency probably play a part in bringing about these fetal movements. Potassium-lack seems to be one of them.

Whether the spontaneous rhythmical movements resulted from firing of neurons or muscle cells alone is open to some question. Faradic stimulation of the central nervous system was ineffectual, but muscles could be induced to contract by placing the electrodes directly over them; even so, the peripheral motor nerves may have been stimulated, for they are present early in development (Windle, 1937). If the occurrence of the movements in question were dependent upon ionic deficiency in the nerve cell itself, one would see no reaction until time sufficient for diffusion from the spinal cord had elapsed, and the great muscle masses of the trunk should be activated as soon and as often as the smaller, more distal muscles. This, very definitely, was not the case; distal movements were in the majority and were nearly always the first to appear. In fact, the very tip of the tail or the hand or foot were usually the sites of first activity. These were the thinnest parts, where diffusion could occur most easily. It is probable that peripheral motor mechanisms were involved.

The greater facility of ionic diffusion from thin parts of the embryo most recently developed may explain an apparent recapitulation of activity in the inverse order of normal development. However, in a series of sufficient length, instances will be found in which forelimbs move before the tail does, and head movements even may precede those of other parts.

CONCLUSIONS

1. Spontaneous contractions of skeletal muscles occurred in cat fetuses 13.5-55 mm. C.R. length placed in physiological saline solutions lacking calcium, potassium, or both. They occurred in isotonic sodium hexametaphosphate and in distilled water. They failed to appear in amniotic fluid and were rarely seen in Locke's solution.
2. Deficiency of calcium and potassium in the surrounding medium appears to be responsible for their appearance.
3. The reaction seems to take place in the peripheral mechanism rather than in the central nervous system. It may involve the skeletal muscles alone or the peripheral motor nerves or nerve endings.

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THE INTERACTIONS OF THE SLOW AND THE FAST CONTRACTION OF CRUSTACEAN MUSCLE

(Five figures)

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IN PREVIOUS publications (van Harreveld and Wiersma, 1936, and Wiersma and van Harreveld, 1938a) it has been shown that the adductor muscle of the chelipeds and walking legs of all decapod crustaceans so far investigated have a double motor innervation. They are innervated by only two motor axons, the stimulation of each of which causes a specific kind of contraction. The contraction caused by the stimulation of the thicker of the motor fibers is the fast contraction, and for this reason this nerve fiber will be called the "fast" fiber; and, correspondingly, the thinner fiber will be called the "slow" fiber, since it causes a slow contraction. Physiological and anatomical evidence shows that both the slow and the fast contraction can occur in the same muscle fiber (Wiersma and van Harreveld, 1938b; van Harreveld, 1938).

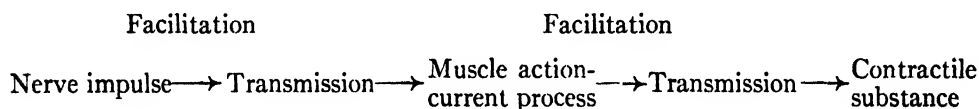


FIG. 1

A series of intermediary reactions can be distinguished in the transmission between the nerve impulse and the mechanical contraction (Marmont and Wiersma, 1938); a scheme for these various steps is given in Figure 1. Though all the steps can be demonstrated in both kinds of contraction, their relative magnitude differs. In marine crustaceans the action-current facilitation is pronounced in both kinds of contraction, whereas in *Cambarus clarkii* this facilitation is demonstrable only in special circumstances (Wiersma and van Harreveld, 1938b). In the same papers experiments have been described on the relation between the size of the muscle-action current and the mechanical contraction which made it clear that a second facilitation process exists between the action current and the mechanical contraction.

The process underlying the muscle-action current seems not to be propagated, nor all-or-none (Wiersma and van Harreveld, 1938b). The distribution of the excitation over the muscle fiber is not a function of the action-current process but is caused by the multiple innervation of the muscle fibers (van Harreveld, 1938). From anatomical evidence it is likely that the endings of the slow and the fast nerve fibers are intermingled on the surface of the muscle fiber.

These considerations make a mutual influence of the two kinds of contraction likely. In the following experiments the influence of fatigue of one kind of contraction on the other and the facilitation of one by the other were studied both with regard to the mechanogram and the action currents.

METHOD

The nerve fibers for the slow and for the fast contractions were prepared by splitting the nerve bundles, as has been described previously (van Harreveld and Wiersma, 1936). Each nerve fiber was stimulated by a separate pair of micromanipulated electrodes. The fiber eliciting the test contraction was usually stimulated at a low frequency, using a thyatron stimulating device which supplied rectangular currents of a short duration. The other fiber was stimulated faradically with an induction coil.

A. INFLUENCE OF THE FAST ON THE SLOW CONTRACTION

Cambarus clarkii and *Astacus trowbridgii*.—To investigate the influence of the fast on the slow contraction, the slow fiber was stimulated at a low frequency (10–20 per second) for periods of 3 seconds at regular intervals of 3 minutes. This rest of 3 minutes between two stimulations is enough to abolish the facilitation caused by the foregoing stimulation of the same fiber (autofacilitation). Accordingly, these contractions were very constant in size and speed; the only variation which ever occurred was a very gradual decline with the aging of the preparation. After a few contractions were obtained in this way, the stimulation of the slow fiber was preceded by a stimulation of the fast fiber. The best results were obtained by stimulating the fast fiber for 30 seconds (40–50 per second), with an interval of 15 seconds before the next stimulation of the slow fiber, to give the muscle time to relax from the fast contraction.

Two types of result were obtained. In a minority of cases (about 20 per cent) the faradization of the fast fiber caused at the first trial a marked depression of the following slow contraction. This depression was of a more or less permanent nature, for only a slight recovery was observed during the following sequence of test stimulations of the slow fiber. Repetition of the stimulation of the fast fiber resulted in a still more pronounced depression of the slow contraction, and in this way the slow contraction could be quite abolished for the frequency of stimulation used. On increasing the stimulation frequency, a slow contraction could be elicited again. We will discuss the nature of this depression later.

In the majority of the cases, instead of a depression there was a facilitation of the slow test contractions which followed the stimulation of the fast fiber (heterofacilitation). This facilitated contraction had a shorter latent period and a quicker rise than a non-facilitated one and usually attained a greater height. When, instead of heterofacilitation, autofacilitation was produced by a stimulation of the same duration and frequency (40–50 per second), the test contraction was much more facilitated. This was even true when the frequency of the stimulus which caused the autofacilitation was the same as that used for the test contraction (10–20 per second). Figure 2 illustrates these phenomena. In a few preparations which initially showed a clear heterofacilitation, this diminished with repetition of the experiment and made way for the depression described above. In some others the facilitation was very poor at the first trial, and in the second trial, depression was obtained.

After complete exhaustion of the fast contraction, faradic stimulation of the slow fiber elicits a contraction which is not noticeable smaller than a similar contraction preceding the exhaustion of the fast contraction.

Marine crustaceans.—A large number of experiments has been performed on *Cancer anthonyi*, *Pagurus ochotensis*, and *Loxorhynchus grandis*; the results conformed with those obtained in *Cambarus* and *Astacus*. In all these species heterofacilitation was ob-

tained; but autofacilitation was more pronounced, the reduction caused by a preceding stimulation of the fast fiber was occasionally found, and exhaustion of the fast contraction did not reduce the height of the following slow contraction. Small quantitative differences were found between the species investigated. For instance, in *Pagurus* the difference between auto- and heterofacilitation is relatively small because of strong heterofacilitation; the reduction by a preceding stimulation of the fast fiber was particularly pronounced in *Cancer*.

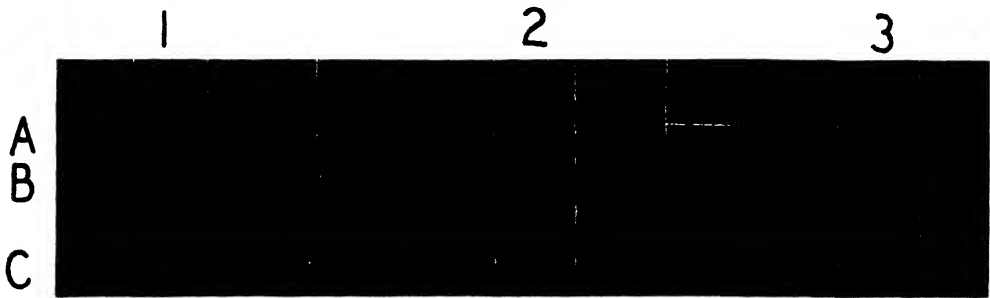


FIG. 2.—Heterofacilitation and autofacilitation of the slow contraction in *Cambarus*. The slow fiber is stimulated at a frequency of 15 per second for periods of 3 seconds with intervals of 3 minutes. In A3 and B2 the test stimulation is preceded by a faradic stimulation of the fast fiber for 30 seconds. Both heterofacilitated contractions are higher and steeper than the normal ones. In C2 the test stimulation was preceded by a stimulation of the slow fiber for 30 seconds at a frequency of 15 per second; a distinct autofacilitation is visible.

B. INFLUENCE OF THE SLOW ON THE FAST CONTRACTION

Cambarus and Astacus.—After complete exhaustion of the slow contraction, faradic stimulation was applied to the fast fiber. The resulting contraction was not noticeably smaller than a similar contraction elicited in the fresh preparation; nor was it possible to suppress in this way the twitch contraction caused by a single induction shock applied to the fast fiber. Because it takes 10–15 minutes to exhaust the slow contraction, the reduction of the twitch which usually occurs during this period cannot be referred with certainty to an influence of the exhaustion of the slow contraction, since such a reduction can also take place by a decline of the twitch during this lapse of time without stimulation of the slow fiber.

To investigate whether the slow contraction has a facilitating influence on the fast, the latter had to be brought into a condition in which such a facilitation would be demonstrable. Under normal circumstances the twitch contraction is so strong that the small increase which might be expected from heterofacilitation would be hard to demonstrate. In previous communications (Wiersma, 1933; Wiersma and van Harreveld, 1938b) it has been stated that the twitch elicited by one single nerve impulse in the fast fiber diminishes gradually on repetition of the stimulus, ultimately disappearing completely (without an appreciable change in the action current). When the twitch contraction has disappeared, faradic stimulation of the fast fiber invariably elicits a large mechanical response, showing that autofacilitation is pronounced under these circumstances. This autofacilitation was further studied in the following experiment. The fast fiber is stimulated with shocks at a frequency of about 1 per second until the twitch has entirely disappeared. Then the



A B

FIG. 3.—Autofacilitation of the fast contraction in *Cambarus*. At A the fast fiber was stimulated with 10 single induction shocks (1 per second); only on the first two is a visible contraction obtained. At B the frequency was suddenly increased to 40 per second for a period of 3 seconds. After 1 second the stimulation was resumed at a frequency of 1 per second; high twitches resulted, which gradually declined.

scribed in the preceding experiment. After reducing the twitch contraction by repeated stimuli, short series (e.g., five) of stimuli at a frequency of one every 2 seconds were given with intervals of 1 minute. It was found that the height of the contractions thus elicited soon became fairly constant (usually with a gradual slow decrease with time). Each series consisted of a first, rather high contraction, followed by a much smaller, second one; whereas the later stimuli either elicited very small contractions or had no visible effect at all. After a fairly constant height of the different contractions was established, a period of 30 seconds of faradic stimulation of the slow fiber was given during the period of rest of the fast fiber, ending 15 seconds before the next series of stimuli was due. The effect of such a stimulation was, in almost every case, a very noticeable increase in the height of the following twitches (Fig. 4). Sometimes the facilitation was clearest in the first, some-

frequency is suddenly increased to about 50 per second for a duration of a few seconds, eliciting a tetanic contraction. Stimulation is then stopped for different periods and afterward resumed with a frequency of 1 per second. It was now found that such shocks will elicit contractions when started within a period of about 30 seconds after the high-frequency stimulation. This experiment is illustrated in Figure 3. The height to which the twitches are facilitated depends on a number of factors. If the duration of the high-frequency stimulation is too short, the effect is small; if it is too long, no effect at all is obtained, probably because of a preponderance of fatigue. In a certain preparation, for instance, only very small twitches were obtained 10 seconds after a high-frequency stimulation of 1 second; no contraction at all resulted after a similar 15-second stimulation period; but after a stimulation period of 5 seconds, large twitches occurred. In all cases the facilitating influence of the high-frequency stimulation becomes gradually smaller as the experiment is repeated.

To investigate the heterofacilitation the preparation was brought into a condition similar to the one de-

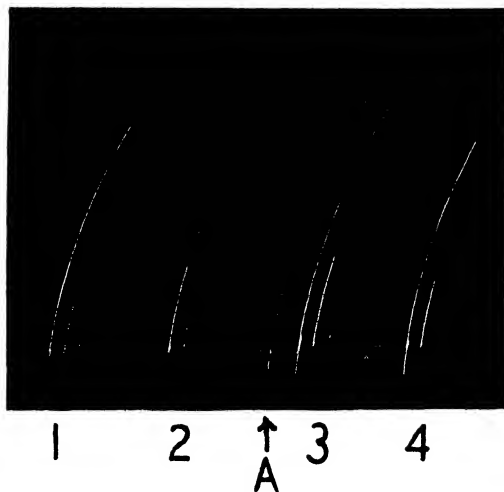


FIG. 4.—Heterofacilitation of the fast contraction in *Cambarus*. Four series of five stimuli applied to the fast fiber at intervals of 1 minute are shown. Only the first three stimuli of each series give a visible effect. The third series is preceded by a faradic stimulation of the slow fiber for 30 seconds. This contraction is recorded at A (drum stopped). All three visible contractions elicited by the next stimulation series are obviously facilitated. This heterofacilitation has almost completely disappeared in the fourth series of stimuli.

times in a later, member of the heterofacilitated row of contractions. Often a member of the stimulation series which did not elicit a visible contraction before the facilitation did so afterward. In a number of experiments the facilitation lasted longer than 1 minute, and sometimes could be detected for a period of 3 minutes.

Marine crustaceans.—Unlike *Cambarus*, *Cancer* and *Loxorhynchus* do not give a twitch contraction on a single nerve impulse in the fast fiber. The tetanic contractions, especially those with lower frequency of stimulation, develop much more gradually than in *Cambarus*, and it is thus possible to apply the same method as has been used to investigate the influence of the fast contraction on the slow. Usually stimulations of the fast fiber with a frequency of about 20 per second were given for a duration of 5 seconds with intervals of 3 minutes. Again the slow fiber was stimulated for a period of 30 seconds, ending 15 seconds before the next test stimulation of the fast fiber was due. Small but very distinct facilitations of the fast contraction by the preceding stimulation of the slow fiber were obtained in both species.

C. MUTUAL INFLUENCE OF THE MUSCLE-ACTION CURRENTS OF THE FAST AND THE SLOW CONTRACTION

A large number of experiments were performed in order to find an influence of the stimulation of one of the motor fibers on the muscle-action currents elicited by the stimulation of the other. *Cambarus*, *Astacus*, and *Cancer* were used in these experiments. The action currents were recorded under the same experimental conditions under which an influence of one kind of contraction on the other was found. Since *Cambarus* and *Astacus* do not usually show any autofacilitation of the action currents of the fast contraction (Wiersma and van Harreveld, 1938a), it could not be expected that they would show any heterofacilitation. In *Cancer* there is pronounced autofacilitation and a marked growth of the action currents during faradic stimulation of the fast fiber, and so heterofacilitation of the action currents might here be expected. However, the results of the experiments were quite negative: the action currents of the fast contraction started and grew in the same way, and to the same extent, before and after a facilitating stimulation of the slow fiber. Also, fatigue of the action currents of the slow contraction had no distinct influence on the action currents of the fast contraction.

In none of the three species was it found that the fast contraction had a significant effect on the action currents of the slow. In a few preparations a slight increase, in some others a slight decrease, of the action currents of the slow contraction following stimulation of the fast fiber seemed to be present. These effects, however, were so small that they cannot be considered as significant, since the preparations sometimes showed spontaneous variations of the same magnitude.

DISCUSSION

Since a distinct heterofacilitation was found for the mechanogram and not for the electrogram under the same circumstances, it is almost certain that the facilitation of the mechanical response obtained is not due to an increase of the action currents but to a facilitation of the transmission process between the action current and the contractile substance (see Fig. 1).

Though the effect of faradic stimulation of the fast fiber was usually a facilitation of the following slow contraction, in a number of cases a depressing influence was observed. In some preparations the facilitating and the depressing effects were even seen in the

course of one experiment. It is likely that a fast contraction has, at the same time, a facilitating and a depressing effect on the slow contraction, and that the result obtained is the sum of these two influences. This is probably one of the reasons for the smallness of the heterofacilitation, compared with the autofacilitation, under similar conditions. Another reason may be that by autofacilitation the height of the action currents is increased (with the exception of those of the fast contraction in *Cambarus* and *Astacus*). Since the action-current process is a link in the chain of events connecting the nerve impulse with the mechanical response, an increase of the action currents will be usually accompanied by an increase of the mechanical contraction (Marmont and Wiersma, 1938).

Facilitation

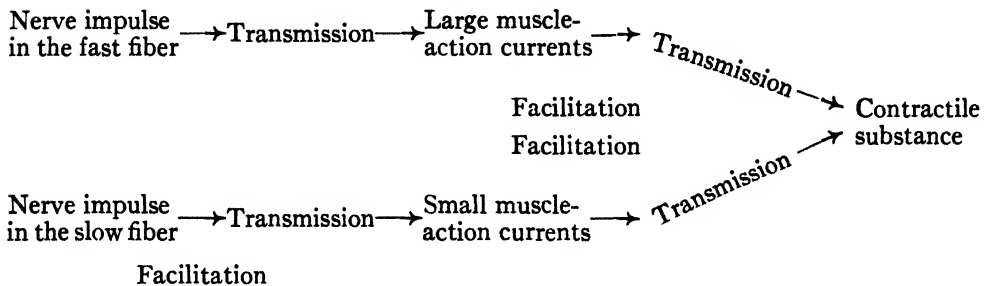


FIG. 5

Since heterofacilitation of the action currents was not found, it must be concluded that the first part of the chain of intermediary processes of the slow and of the fast contraction, including the process underlying the action currents, are independent to such an extent that they even do not influence each other. This means that the intermediary processes of the fast and the slow contraction, including the action currents, are locally quite separated. From anatomical evidence it is likely that the numerous endings of the fast and those of the slow fiber do show local separation, which makes the separation of the beginning of the two chains of intermediary processes possible.

There are several arguments for the belief that the same contractile substance is involved in both kinds of contraction. It has been shown (van Harreveld and Wiersma, 1936) that in the adductor muscle of *Cambarus* the maximum tension which can be developed by each of the two kinds of contractions is approximately the same. It has been found, furthermore (unpublished), that the chemical changes in the muscle caused by the two kinds of contraction are qualitatively the same and about equal in magnitude when the two contractions are comparable in strength and duration. The fact that exhaustion of one of the contractions does not noticeably decrease the other seems opposed to this view. It has been shown, however, that during such stimulation the action currents diminish long before the contraction has reached its maximum, and that they become so small when the contraction starts to decline that this decline is most likely due to the fatigue of the action current (Wiersma and van Harreveld, 1938b). Therefore, it would not be possible to exhaust the contractile substance by faradic stimulation of the motor axons.

It has been mentioned that the twitch contraction in *Astacus* and *Cambarus* can disappear without a reduction of the action current or a fatigue of the contractile substance. It must be concluded that this depression is due to a change in the transmission process between the action current and the contractile substance. The depression, caused by a short faradic stimulation (30 per second) of the fast fiber, of a slow contraction elicited by low-frequency stimulation must be due to a similar block, since the action currents were not noticeably influenced by the preceding fast contraction.

The question whether the transmission processes between both action currents and the contractile substance are separated or common cannot be answered. Since these transmission processes can be mutually heterofacilitated, it seems certain that they are qualitatively the same, which is further supported by the fact that both transmission processes can be blocked by the same inhibitory fiber (Marmont and Wiersma, 1938; Wiersma and Marmont, 1938). A scheme of the intermediary processes involved in the double motor innervation is given in Figure 5.

SUMMARY

The mutual influence of the slow and the fast contractions of the adductor muscle of the chelipeds and walking legs of several fresh-water and marine crustaceans was studied.

Exhaustion of one kind of contraction did not decrease the action currents of the other; nor did one contraction produce a detectable facilitation of the action currents of the other.

Exhaustion of one kind of contraction did not noticeably reduce the mechanical response of the other. A distinct, but small facilitation of the mechanical response by a preceding faradic stimulation of the other motor fiber (heterofacilitation) was found.

It is concluded that the first parts of the chain of intermediary processes between the nerve impulse and the mechanical contraction, including the action currents, are locally separated in the two kinds of contraction, and that the heterofacilitation is located in the transmission process between the action current and the contraction. It is argued that the contractile substance is the same for both kinds of contraction.

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THE RESPIRATORY METABOLISM OF STARVED JAPANESE BEETLE LARVAE (*POPILLIA JAPONICA* NEWMAN) AT DIFFERENT RELATIVE HUMIDITIES¹

(One figure)

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THIRD-INSTAR larvae of the Japanese beetle, *Popillia japonica*, were starved at different humidities by Ludwig and Landsman (1937), who found that the length of life varied directly with the humidity. At a low humidity of 0-5 per cent the larvae lived about 4 days, while at a high humidity of 96 per cent they lived about a month. It was also noted that their water content at death was very nearly constant (56-58 per cent), regardless of the humidity, indicating that the larvae died of desiccation. The only exception was found in the high humidity, where the larvae survived for a month and had a much higher water content (68 per cent) when they died. Hence, at this humidity death was due not to desiccation but probably to starvation.

Ludwig (1936) observed that Japanese beetle larvae lose water rapidly at the beginning of the desiccation period and that the rate of water loss progressively decreases. It was also noticed that a decrease in the activity of the larvae occurred and that they became quiescent after the first day. He suggested that the decrease in the rate of water loss after the first few days of desiccation may be associated with a decrease in the metabolic rate accompanying the quiescent condition. The relatively slow rates at which the prepupal and pupal stages become desiccated also support this view.

The effect of desiccation on metabolic rate differs in different insects. Caldwell (1925) found an increase in metabolism when the larvae of the mealworm, *Tenebrio molitor*, were desiccated to less than one-half of the possible limit. Continued desiccation caused it to fall far below normal. He suggested that desiccation had a stimulating effect on the metabolic rate. In the same paper Caldwell reported that under the same conditions the eight-spotted forester moth, *Alpia octomaculata*, did not show an increased metabolic rate. In this form it drops steadily to a low point when death occurs. Bodine (1933) subjected eggs of the grasshopper, *Melanoplus differentialis*, to hypertonic solutions and found a decrease in the oxygen consumption at all stages of desiccation. Ludwig (1937) observed a decrease in the metabolic rate when the nymphs of the grasshopper, *Chorthippa viridifasciata*, were starved at different relative humidities. In these experiments starvation caused death in 5 or 6 days, regardless of the humidity. Moreover, the degree of humidity exerts no significant influence on the rate of oxygen consumption, although at the high humidities water content increased to 82.4 per cent and at the low humidities it decreased to 58.8 per cent. Water content, therefore, had no influence on metabolic rate; but starvation caused a rapid decrease in metabolism, which became progressively lower until death. Since Japanese beetle larvae can withstand periods of

¹ The present experiments were undertaken at the suggestion of Dr. Daniel Ludwig, to whom the writer wishes to express his deep appreciation for the advice and generous assistance given him throughout the course of these investigations.

starvation for as long as a month when maintained in an atmosphere of high humidity, it is very evident that their type of response must be different from that of *Chortophaga*. Hence, an experiment was devised to investigate changes in metabolism accompanying starvation of Japanese beetle larvae at different relative humidities.

MATERIALS AND METHODS

Third-instar larvae of the Japanese beetle were collected in the field during the fall and early winter. Each individual was placed in a 1-ounce metal salve box containing moist soil of approximately the same humidity as the normal environment of the larvae. They were then stored in a refrigerator, where a low temperature of 8–10° C. prevented development and insured a ready supply of larvae during the winter months. Each larva was examined at intervals of several days. A few grains of wheat were added to serve as food, and sufficient water was added to maintain the proper humidity. Before subjecting the larvae to experimental conditions, they were removed from the refrigerator and kept at room temperature for a period of a week to acclimate them to the higher temperatures used in the experiment.

During the experiments the larvae, exclusive of fecal materials, were weighed each day on a chainomatic balance sensitive to 0.1 mg. Respiratory metabolism was measured by the modified Krogh manometer (Bodine and Orr, 1925). Readings were taken at a constant temperature of 25° C. Each larva was hung inside the manometer bulb in a tight-fitting wire basket to prevent excess movement. To determine oxygen consumption, 2 cc. of 2 per cent KOH were placed in the manometer bulbs to absorb the carbon dioxide. Readings were then taken over a period of several hours, after which the KOH was removed and the bulbs thoroughly washed. The manometers were then returned to the water bath, and the readings were again taken for several hours. From the difference between the first and the second sets of readings the carbon dioxide production and the respiratory quotients were determined. Readings of the respiratory metabolism were made on larvae exposed to relative humidities of 0–5, 56, and 96 per cent. These values were obtained by using the following substances in the bases of ordinary desiccators: for 0–5 per cent, anhydrous calcium chloride; for 56 per cent, a saturated solution of sodium bromide; and for 96 per cent, distilled water (Ludwig, 1937). The larvae used for the metabolism experiments were kept in the desiccators at the various humidities except for the daily period of 4 or 5 hours required for the respiratory readings. This daily exposure to the humidity of the manometer flasks probably did not materially change the water content of the tissues, since complete desiccation at the time of death showed approximately the same residual water as found by Ludwig and Landsman (1937) for individuals not used in the respiratory experiments. The desiccators were always kept at a constant temperature of 25° C. Only during the period required for weighing and adjustment in the manometers were the larvae exposed to laboratory temperatures. All experimental computations and conclusions in these experiments are based on data obtained from twenty or more larvae.

OBSERVATIONS

When the third-instar larvae of the Japanese beetle were starved at the humidities of 0–5, 56, and 96 per cent, they suffered a rapid loss in weight during the first day. At low humidities this rapid decrease continued until the larvae died; but at the higher humidities the rate decreased gradually, and finally there was a rather constant, small,

daily weight loss. At humidities of 0-5 and 56 per cent the larvae died with approximately the same water content (53.2-54.5 per cent), while at the high humidity of 96 per cent individuals died when their water content had decreased to only 75.9 per cent.

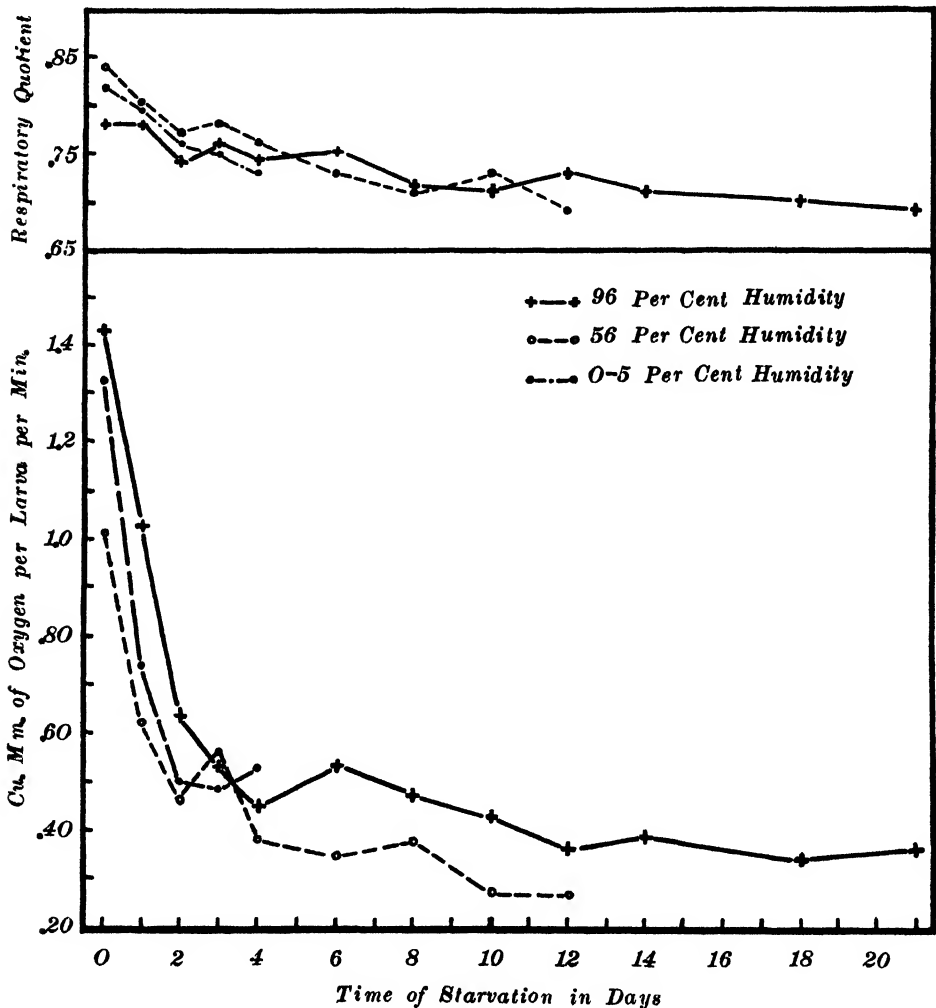


FIG. 1.—Changes in the metabolic rate of Japanese beetle larvae starved at different relative humidities.

Larvae at 0-5 and 56 per cent humidity died of desiccation in an average of 4 and 12 days, respectively; while at 96 per cent they died of starvation in an average of 26 days. The decrease in metabolic rate which accompanied starvation at each humidity is plotted in Figure 1. The amount of oxygen consumed by each larva decreased rapidly at the beginning of the starvation period. This rapid decrease, however, did not continue and after the second day metabolism remained at a low level, with very little

change until death. A drop in the respiratory quotient was also observed. Starting at an average value of 0.82, a rapid decrease occurred for several days, and then the res-

TABLE 1
THE EFFECT OF HUMIDITY ON THE RESPIRATORY EXCHANGE OF
STARVED JAPANESE BEETLE LARVAE

Hours of Starvation	Weight as Percentage of Original Weight	Water Content (Per Cent)*	O ₂ Mm ³ /Min / Larva	O ₂ per Larva as Percentage of Original	O ₂ Mm ³ /Min /Gram	Percentage of Decrease in O ₂ per Gram	R Q
Calcium Chloride, 0-5 per Cent Humidity							
0	100 0	81 0	1 325±0 08	100 0	4 54	00 0	0 82
24	76 0	75 3	0 740±0 04	55 8	4 36	4 0	0 80
48	64 5	68 9	0 507±0 03	38 3	3 52	22 0	0 76
72	52 0	62 9	0 490±0 03	36 9	4 04	12 0	0 75
96	51 2	55 9	0 546±0 03	41 2	4 72	Above	0 73
Sodium Bromide, 56 per Cent Humidity							
0	100 0	81 0	1 006±0 03	100 0	5 15	00 0	0 84
24	85 0	78 5	0 623±0 03	62 0	3 87	23 8	0 80
48	74 2	76 5	0 477±0 03	47 0	3 20	37 5	0 77
72	68 5	74 6	0 566±0 04	56 2	2 79	45 5	0 78
96	68 7	72 6	0 381±0 03	37 9	2 90	43 4	0 76
144	55 0	68 9	0 344±0 05	34 2	2 61	47 6	0 73
192	52 8	64 9	0 370±0 03	36 8	2 91	43 2	0 71
240	50 0	60 8	0 277±0 03	27 5	2 77	46 0	0 73
288			0 269±0 03	26 5	1 81	64 6	0 69
Distilled Water 96 per Cent Humidity							
0	100 0	81 0	1 434±0 07	100 0	6 55	00 0	0 78
24	91 5		1 076±0 04	75 2	5 41	17 5	0 78
48	87 5	80 0	0 621±0 02	43 4	3 18	51 6	0 74
72	82 8		0 557±0 03	38 9	2 95	55 2	0 76
96	82 0		0 452±0 08	31 5	2 42	63 4	0 74
144	79 5		0 544±0 06	38 0	3 08	52 9	0 75
192	77 5	78 9	0 472±0 08	33 0	2 76	57 8	0 71
240	79 8		0 438±0 01	30 6	2 45	62 6	0 71
288	74 5		0 368±0 02	25 7	2 49	62 0	0 73
336			0 389±0 03	27 2	2 22	66 1	0 71
432		77 3	0 345±0 02	24 1	1 92	68 8	0 70
504			0 307±0 02	25 7	2 23	70 7	0 69
576			0 340±0 06	23 8	1 92	71 0	0 71

* The data in this column were obtained from unpublished work of Ludwig

piratory quotient fluctuated about 0.70 and remained at this value until death. Calculations were also made of the oxygen used per gram per minute. These results show the same trend as those shown in Figure 1, except at the humidity of 0-5 per cent, where an

apparent increase in the metabolic rate is indicated after the second day. However, this apparent increase was caused by the very rapid loss in weight which accompanied starvation at the low humidity.

Table 1 contains a tabulation of the weight and water loss, the rates of oxygen consumption, the respiratory quotients, and the percentage of decrease in respiratory metabolism at different periods of starvation for each humidity. This table demonstrates a steady decrease in the metabolic rate regardless of the humidity or the water content of the larvae.

Table 2 shows that the metabolic rates after comparable hours of starvation at different humidities are, in most cases, not statistically different. However, it was observed

TABLE 2
PROBABLE SIGNIFICANCE OF DIFFERENT RESPIRATORY READINGS OBTAINED
AFTER VARIOUS PERIODS OF STARVATION AT DIFFERENT HUMIDITIES

Oxygen Consumption per Larva per Minute	Difference between Means	Probable Error of Dif- ference	Difference/ Probable Error of Difference
48 hours at 0.5 and 56 per cent	0 030	0 049	0 6
48 hours at 0.5 and 96 per cent	0 114	0 040	2 8
48 hours at 56 and 96 per cent	0 144	0 037	3 9
72 hours at 0.5 and 56 per cent	0 076	0 055	1 4
72 hours at 0.5 and 96 per cent	0 067	0 043	1 6
72 hours at 56 and 96 per cent	0 009	0 053	0 2
96 hours at 0.5 and 56 per cent	0 165	0 044	3 7
96 hours at 0.5 and 96 per cent	0 094	0 083	1 1
96 hours at 56 and 96 per cent	0 071	0 083	0 8
144 hours at 56 and 96 per cent	0 200	0 024	0 8
192 hours at 56 and 96 per cent	0 102	0 092	1 1
240 hours at 56 and 96 per cent	0 161	0 260	6 2

that in several cases the difference between the means divided by the probable error of the difference between these means gives values of more than 3, indicating that the means are statistically different. Using the 240-hour stage of starvation in both the 56 and 96 per cent humidities, the value is 6.2. However, if reference is made to Table 1, it will be noticed that at 56 per cent humidity the metabolic rate was reduced 27.5 per cent of its original value, and that at 96 per cent humidity a reduction to 33.0 per cent occurred. This table also shows that the individuals kept at 56 per cent humidity used less oxygen per minute than those kept at 96 per cent humidity, and this difference was maintained throughout the experiment. In each case where the difference divided by the probable error of the difference gives values of more than 3, the means are statistically different, because the small oxygen consumption of small individuals (those used at the 56 per cent humidity) was compared with larger values observed for the larger individuals kept at 96 and 0.5 per cent humidities. Larvae approximately the same size never showed a statistical difference in the rate of oxygen consumption after comparable hours of starvation, regardless of the humidity. Therefore, water content of the larvae

seems to have no effect on the metabolic rate, and the decrease observed is caused by starvation.

DISCUSSION

Different species of insects manifest different susceptibilities to changes in humidity. Some can withstand very low humidities for a long time, while others need a very moist atmosphere for survival. Mellanby (1932a, 1932b) showed that the mealworm, *Tenebrio molitor*, can conserve metabolic water and thus withstand the very dry environment in which it lives. Conversely, Evans (1935) found that the prepupa and the pupa of the blowfly, *Lucilia sericata*, lose a constant amount of dry matter at all humidities, and that the greater loss of weight in dry atmospheres is entirely due to a loss of water. Similarly, Ludwig (1936) has shown that the larvae of the Japanese beetle are unable to conserve water derived from metabolic processes. Hence, in these experiments the weight loss was due almost entirely to a loss of water, except at 96 per cent humidity, where considerable amounts of tissue solids were utilized and where death was caused by starvation (Ludwig and Landsman, 1937). The rapid loss in weight that invariably occurred at the beginning of the experiments decreased gradually in the higher humidities, and finally there was a constant, small, daily weight loss; while at the lower humidity, death occurred before similar results could be observed.

Starvation at different relative humidities always causes a decrease in the respiratory metabolism. Bodine (1921) believed that starvation caused the nymphs of the grasshopper, *C. viridifasciata*, to lose water rapidly, dehydration causing death. However, Ludwig (1937) demonstrated that this form died of starvation just as soon at high as at low humidities, although at the higher value no lowering of the water content was observed. In the grasshopper, utilization of tissue solids was very rapid, and, regardless of the water content of the body, death occurred after an average of 5.2–6.6 days of starvation. From the beginning of the desiccation period the metabolic rates of the grasshopper nymphs decreased very rapidly, and continued to do so until death occurred. In the present experiments it was found that the metabolic rate of the Japanese beetle larvae decreased rapidly for only 2 days and then remained relatively constant at a low level until death.

In these experiments, as in the case of the grasshopper, it was found that the water content of the tissues did not affect the metabolic rate. Water content, however, influenced the time of survival. Because the metabolic rate dropped to a low value, it was possible for the larvae, at high humidities, to live as long as 26 days, finally dying of starvation. At humidities of 0–5 and 56 per cent desiccation caused death by lowering the water content of the body to approximately 54 per cent. The metabolic rates of the grasshoppers did not show this response, and, regardless of the humidity and water content, grasshoppers died of starvation. Ludwig and Landsman (1937) found the time of survival of the Japanese beetle larvae to be slightly longer for the same humidities used in these experiments. These differences may be explained by the greater amount of handling required in measuring the respiratory metabolism.

The natural differences in the type of development and in the feeding habits of the grasshopper and the beetle larva may be correlated with difference responses to starvation. The grasshopper requires constant feeding, and there is little storage of fats. Hence, death by starvation may occur in a short time. On the other hand, the Japanese beetle larva must store enough food for use during the pupal stage. Therefore, it con-

tains large quantities of fats, which may be used under conditions of starvation. The fact that the respiratory quotient of these larvae fell from an average of 0.82 to 0.70 indicates the utilization of fat. The ability of the Japanese beetle larvae to survive starvation for nearly a month at high humidities is probably due to the rapid reduction of the metabolic rate to a low level, thus reducing the consumption of stored foods.

SUMMARY

1. The time of survival of the Japanese beetle larvae depends on the humidity. At 0-5 per cent they lived 4 days; at 56 per cent they lived 12 days; and at 96 per cent they lived 26 days.

2. In these experiments death was caused by starvation at a humidity of 96 per cent and by desiccation at the lower humidities. The larvae exposed to 0-5 per cent humidity died with an average water content of 53.2-54.5 per cent, indicating that death was caused by desiccation; and at 96 per cent humidity they died of starvation, with a water content of 75.9 per cent.

3. Starvation caused the metabolic rate to fall rapidly during the first 2 days and then more slowly, fluctuating about a low level, which was maintained until death. The rapid decrease in the metabolic rate to a low point, barely sustaining life, may be the factor allowing the larvae to live as long as a month at high humidities without food.

4. There is a decrease in the respiratory quotient from an average of 0.82 to 0.70, indicating the oxidation of stored fats during starvation.

5. Different relative humidities or the water content of the larvae have no direct effect on the respiratory metabolism. The observed fall in the rate of oxygen consumption is due to starvation.

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AXIAL SUSCEPTIBILITY TO SERUM ANTIBODIES IN EUPLANARIA

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IN *EUPLANARIA DOROTOCEPHALA* the fundamental polarity of the organism is in some way associated with an anteroposterior differential in susceptibility to cytolytic agents. The mass of evidence at hand permits no serious doubt of this fact. It is also a well-established fact that disintegration may be induced in *E. dorotocephala* by a wide variety of chemical and physical agents and conditions that appear to have nothing in common except the property of causing cytolysis (Child, 1928, 1930; Buchanan, 1930a, 1930b, 1935). It has further been shown that certain metabolic processes decrease in rapidity from anterior toward posterior within the zooids (Child, 1928) and that the ability of the organism to appropriate water varies in a similar fashion (Buchanan, 1930a, 1930b). These facts warrant the conclusion that cytolytic agents which reveal polarity do so by acting upon, or through, an anteroposterior quantitatively graded state of protoplasmic activity. From this line of evidence, then, polarity is associated with, or represented by, an anteroposterior differential in reactivity.

But the analysis of organic polarity still presents many problems. Among these is the question of the role played by protein metabolism. An attack on the problem of the properties of the proteins along the polar axis has been undertaken by one of us (C. A. L.). He proposed to inject into the rabbit antigenic material prepared, as described below, from living *E. dorotocephala*. It was assumed that the planarian proteins might be antigenic and that any antibody generated in the body of the rabbit would be specific for planarian proteins. With these assumptions it would follow that, if protein reactivity along the long axis of the planarian body is uniform or ungraded, cytolysis by such antibodies would occur simultaneously in all regions of the animal, or at least not follow a time progression from anterior toward posterior.

Accordingly, experiments were planned with these objectives. As the work progressed and it became apparent that, in the rabbit, antibodies destructive to planarian tissues may be so induced, the operations were extended to include attempts to identify the nature of the antibodies by testing their temperature stability and by other means, to determine the duration of the response of the rabbit to the planarian antigen, and the action of the antibodies on regeneration in planarian pieces. The present paper is confined to a report of the presence of antiplanarian antibodies in serum from treated rabbits, and the order of cytolytic disintegration in the planarian when subjected to serum from treated rabbits. The work of preparation of the antigens and the sera, and the handling of all experimental material, was performed by the junior author. The senior author co-operated in determining the effects of the sera, made certain other contributions, and prepared this report. In subsequent papers the junior author will report the further progress of the work.

MATERIALS AND METHODS

Healthy rabbits not previously employed for experimental purposes were selected. *Euplanaria dorotocephala* that had been starved from 6 to 15 days were first washed in

several changes of tap water, followed by several washings in sterile 0.85 per cent NaCl. The planarians were then blotted between filter papers, ground in a mortar, and suspended in sterile 0.85 per cent NaCl, then extracted for 30 minutes at 4°–6° C. The suspension was centrifuged at 1,500–2,000 r.p.m. for 10 minutes, and the supernatant liquid used for the injections.

Ordinarily, 4 cc. was given at each injection, with three injections in each rabbit at 3-day intervals. The injections were given intravenously, intraperitoneally, or subcutaneously. Material to be injected intravenously was first filtered through a Berkefeld candle of *N* porosity; but it was found that, when so injected, it was highly toxic. In many cases death, apparently from protein shock, followed within 15 minutes. Accordingly, intraperitoneal or subcutaneous injection was usually employed.

The sera were prepared from blood drawn from the marginal vein of the ear from 4 to 42 days after the last injection of antigenic material. As stated above, only rabbits that had not been previously employed for experiments were injected. Serum prepared from normal healthy uninjected rabbits was used in control experiments.

The experiments reported here are divided into two groups. The first group was designed to test the viability of *E. dorocephala* in the serum of treated rabbits, as compared with viability in the serum of control animals. In the second set of experiments the purpose was to trace the regional differences in susceptibility to the serum of treated rabbits. The general method employed in experiments of the first group was as follows: A series of identical dilutions of serum from normal and from treated rabbits was made up in tap water.¹ Lots of ten *E. dorocephala*, carefully selected with respect to length, state of nutrition, and uniformity of previous care, were subjected in flasks to 50 or 100 cc. of each dilution of serum, both from injected and from normal rabbits. The range of dilutions is shown in Table 1. The solutions of sera were renewed every 24 hours. The time of appearance of the first sign of disintegration was noted for each lot of ten animals, and the course of the disintegrative process was observed at frequent intervals. The approximate time required for complete disintegration of all the planaria in each flask was also recorded. It was soon found that in the various dilutions of serum from normal rabbits no disintegration occurred at room temperature within the time required for complete disintegration of the planarians in the serum from treated rabbits. For this reason data on the controls are omitted from Table 1.

In the second set of experiments, designed to observe more closely the order of disintegration, watch glasses were prepared, each containing a single carefully selected planarian. In each watch glass 5 cc. of 1:40 serum from injected rabbit was poured over the animal, and sketches of its state of disintegration were made from time to time. The medium was changed every 24 hours. These, as well as the first group of experiments, were conducted at room temperature.

RESULTS

The effect of the various concentrations of serum from rabbits injected with the planarian antigen is shown in Table 1. Since no disintegration appeared in planarians treated with serum from normal rabbits under identical conditions of concentration and temperature within the time limits shown in the table, it is clear that the serum of

¹ Much work has shown that distilled water is itself a cytolytic agent (Buchanan, 1930a). In this laboratory, tap water appears to contain the appropriate concentrations of salts for normal maintenance of *E. dorocephala*.

treated rabbits contains an antibody which causes structural breakdown in living planaria. The data also show that the effectiveness of the antibody decreases with increasing dilution; in solutions containing 1 part of serum and 159 parts of tap water (omitted from the table) the cytolytic effect of the antibody is not apparent after 7 days of treatment. An inspection of the table also shows that the cytolytic action is more prompt in serum taken from rabbits recently injected with the antigen than in serum taken after a lapse of time.

The contrast in behavior between animals in the normal serum and those in serum from injected rabbits is quite sharp. Almost immediately on application of the sera,

TABLE 1
DISINTEGRATION OF *Euplanaria* IN VARIOUS CONCENTRATIONS OF SERUM
FROM RABBITS INJECTED WITH *Euplanaria* EXTRACT

EXPT. No.	PLANARIA		SERUM DRAWN DAYS AFT- ER LAST INJEC- TION	1-10		1-20		1-40		1-80	
	Size (Mm.)	Starved (Days)		Disin- tegra- tion Began (Hr.)	Disin- tegra- tion Com- plete (Hr.)	Disin- tegra- tion Began (Hr.)	Disin- tegra- tion Com- plete (Hr.)	Disin- tegra- tion Began (Hr.)	Disin- tegra- tion Com- plete (Hr.)	Disin- tegra- tion Began (Hr.)	Disin- tegra- tion Complete (Hr.)
I	12-18	14	17	.	..	60	72	60	84	70	98
II-1	19-20	5	6 and 8	24	52	37	52	44	84	84	68% intact in 168
II-4	12-15	7	14	23	49	23	61	36	97	None in 97
II-9	14-17	27	42	20	48	24	94	94	148	None in 148
III-2	12-15	12	10	44	80	48	114	80	150	None in 148
IV-1	12-15	7	4	41	72	48	96	68	140	96	30% intact in 170

those in the serum from treated rabbits twist about and show all signs of being irritated. Later they become less active than animals in normal serum; if inverted, they right themselves but do not move about. Much later they assume a curved position, with the dorsal surface convex. Shortly after this position is assumed, disintegration begins. No such behavior is noted among animals subjected to the same concentrations of the normal serum. Although, somewhat less active than in tap water, they otherwise behave quite normally.

The order of disintegration was observed in the watch-glass experiments with the aid of a hand lens or a binocular microscope. In all, eighty specimens were studied. In sixty-five cases disintegration began at the extreme anterior end. After the erosion of about one-fourth of the animal, proceeding from anterior toward posterior, disintegration began at the extreme posterior end. Complete disintegration was reached when that proceeding from anterior toward posterior met with the disintegration proceeding anteriorly, invariably in the region just posterior to the mouth. Of the remaining fifteen, several exceptionally long animals began to disintegrate at the posterior end before

anterior disintegration appeared. In others, also long animals, some disintegration occurred in the vicinity of the mouth before anterior disintegration began. The general picture of the disintegrative process in these animals treated with serum from injected rabbits was very similar to that observed during disintegration by potassium cyanide, distilled water, absence of oxygen, and other cytolytic agents as described by other observers.

It has been shown that certain cytolytic agents are ineffective in the presence of hypertonic solutions (Child, 1930; Buchanan, 1930b). In the present work the osmotic pressure of the various concentrations of sera was not determined. But there is no reason to believe that the differences observed between the effects of normal and experimental sera may be due to differences in osmotic pressure. The osmotic pressure of rabbit blood is given as 6.809 atmospheres. The highest concentration employed here was 1 part serum and 9 parts water, and comparisons were made between identical concentrations of normal and experimental sera. No doubt such solutions are distinctly hypertonic, but they are not sufficiently so to prevent the water uptake associated with cytolysis.

DISCUSSION

We have to consider, first, the fact of cytolysis by the antibodies in the serum from treated rabbits, and second, the fact that this cytolysis begins almost invariably at the anterior end of the planarian and proceeds posteriorly before the posterior end begins to disintegrate. Thus, so far as the time factor is concerned, all regions of the animal are not equally susceptible to the cytolytic agent.

Evidence, to be presented in some detail in later papers, indicates that the anti-substance in the serum of treated rabbits is protein; at least it is as heat-sensitive as are the proteins. But data associating it with a specific fraction are not yet available. In the absence of evidence to the contrary we may follow the conventional interpretation of the nature of the action of antigenic materials and assume that planarian proteins, or certain of them, induce in the rabbit the production of precipitins,² probably protein in character. In the present state of the work it is not possible to state whether one or several precipitins occur in the serum from treated rabbits. Until analyses now in progress are more complete, it may be assumed that the observed cytolysis is a consequence of the precipitation of planarian proteins by the antibodies.

There is also insufficient knowledge at hand as to whether the action of the antibodies on planarian proteins is due to a specific chemical interchange or to the adsorption of the one by the other. In either event it is quite clear that those substances, presumably proteins, which are acted upon by the antibodies are more reactive at the anterior end of the animal than elsewhere and that the reactivity decreases uniformly from each end toward the region immediately posterior to the mouth, where reactivity is least and cytolysis occurs last.

In specimens of *E. dorotocephala* as long as those employed here, the body consists of more than one zooid (Child, 1930). The posterior limit of the most anterior zooid is approximately that region last to undergo cytolysis. When the order of disintegration by the ant substance is considered in the light of this fact, it becomes clear that the

² Heating to 55°-56° did not destroy the planariacidal properties of the serum from treated rabbits, but precipitation tests with planaria extracts have not yet been completed.

anterior zooid is characterized by a gradient in reactivity of the proteins, or certain of them.

The imposing mass of data concerning the graded differential in protoplasmic activity associated with the fundamental polarity of *E. dorotocephala* has been reviewed elsewhere (Child, 1928, 1930; Buchanan, 1930a, 1930b, 1935). It includes evidence of gradients in susceptibility to lipid solvents, to high temperature, to anesthetics and other depressants, to lack of oxygen, to calcium precipitants, to hydron concentration, in ability to appropriate water, in carbon dioxide production and oxygen utilization, and other evidences of protoplasmic activity. All lines of evidence agree in indicating that the anteroposterior axis of the zooids is characterized by an anteroposterior decrease in rate of metabolic processes. The present contribution adds the fact that the reactivity of the proteins also decreases from anterior toward posterior. Several interpretations of this fact are possible. It is difficult to assume that the antibodies attack an antero-posterior series of different proteins, each progressively less reactive than the next anterior one, for this involves assuming that the serum from treated rabbits contains an almost infinitely great number of antibodies or that a single antibody is nonspecific. On the other hand, assuming the antistubstance to be highly specific, as is usually the case with immunity bodies, we have to account for the fact that it attacks the proteins of the anterior region first. Either protoplasmic structure is increasingly less dependent upon proteins anteroposteriorly or the proteins are increasingly more protected from precipitins from anterior toward posterior. Work now under way is planned to determine the nature of the proteins which show this differential.

The question as to which, if any, of the many metabolic processes which show this differential in rate along the polar axis is the master reaction upon which organic polarity depends remains unanswered. The line of approach indicated here holds out promise of important contributions to a more complete understanding of the nature of the metabolic gradient and, through it, of the nature of organic polarity.

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THE EFFECTS OF SUPERSONIC VIBRATIONS ON RECONSTITUTION AND HEAD FREQUENCY IN EUPLANARIA DOROTOCEPHALA¹

(Five figures)

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MANY experiments have been performed dealing with susceptibility, reconstitution, and the frequency of the regeneration of heads in relation to a large number of physical and chemical agents using the fresh-water flatworms as experimental animals. As regards the words "regeneration" and "reconstitution," the use of both is justified. The head and posterior end *regenerate*, but the piece as a whole undergoes *reconstitution* into a whole animal. Regeneration is one feature of reconstitution. Properly used, the two words do not mean the same thing, though many use the word "regeneration" for all reconstitution. Such agents as dilute KCN (Child, 1916), anesthetics (Buchanan, 1922), caffeine (Hinrichs, 1924), mechanical stimulation (Child, 1920), and changes in temperature conditions influence reconstitution.

Two factors are concerned in head regeneration in *Euplanaria dorotocephala* (Child and Watanabe, 1935): the physiological condition of the cells directly concerned, and the physiological condition of other regions of the piece posterior to the region of the developing head. Other works (Child, 1916; Behre, 1918; Buchanan, 1922; and Hinrichs, 1924) also have shown that inhibiting agents acting on the head-forming cells tend to decrease head frequency. The action of such agents on other parts of pieces cut from *Euplanaria* tend to increase head frequency. The results depend on the relation between these two factors and the period of exposure to the agent.

By "head frequency" we mean the frequency of the various head forms that have reconstituted from lots consisting of pieces of a certain length and from a certain body-level of animals of the same length. If a given lot of pieces shows a majority of normal or near-normal animals, the lot is characterized as having a high head frequency. A lot giving a majority of headless or strongly inhibited head forms is characterized as having a low head frequency.

Wiercinski and Child (1936) obtained evidence which indicated that supersonic vibrations, primarily a mechanical factor, produced the disintegration gradients such as had been observed with other agents. These results suggested the possibility that supersonics might affect head frequency.

MATERIALS AND METHODS

Euplanaria dorotocephala, found in freshwater springs near Chicago, was used as the experimental animal. The worms were kept in the laboratory in large granite pans filled with well water, were fed liver twice a week, and the water was changed five times a week, care being taken to remove all liver debris.

Before experimentation several pans of worms were kept without food for 2 weeks,

¹ The author wishes to thank Professor C. M. Child for advice and criticism received throughout the progress of this work.

since this procedure has been found to give greater uniformity in experiment. Lots of 60 animals each, of uniform size, were selected from the starved stock for the control and experimental groups. For head-frequency studies the animals were cut in transverse sections, as shown in Figure 1. Only the anterior zooid was used, and the pieces were approximately one-eighth the length of the animal, exclusive of the head. Of the 60 pieces cut from each region, those that showed the greatest variation in length were discarded. In the majority of cases, 50 pieces were used. This procedure decreased any error that might be attributed to the difference in length of the pieces. The test and control animals were sectioned in well water. The test pieces were placed on the quartz crystal with a wide-mouth pipette, either immediately after section or after periods of delay, were exposed to supersonic vibrations, and were returned to well water immediately after exposure. Child (1916) described five classes of regenerated pieces, depending on the degree of inhibition, and these have been commonly used in such studies since that time; hence, the definition of each class can be omitted here. The classes and the arbitrary values assigned to each type of head were: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; and acephalic, 20. To obtain the "head-frequency index," these arbitrary values were multiplied by the frequency of each type of heads; and the sum of all was divided by the total number of living animals. The "mean," described by Child and Watanabe (1935), is identical with the foregoing head-frequency index.

The apparatus employed is a Hartley oscillator. The appendix in a paper by Harvey, Harvey, and Loomis (1928) gives the essentials for constructing the oscillator. The supersonic vibrations were produced by activating an octagonal piezoelectric quartz crystal 5.0 mm. in thickness and 4.5 mm. in width and length, giving a natural frequency of 612 kilocycles per second. The oscillating circuit is tuned to approximately the frequency of the crystal.

The crystal was mounted between two brass electrodes cut and milled from a $\frac{3}{8}$ -inch brass plate of the same length and width as the crystal. This was set on the stage of a grounded microscope. For observation of supersonic phenomena a lower electrode was used, which had a circular opening to permit reflected light to come through. For exposing pieces of worms a solid electrode was used. The upper electrode is cut in the shape of a frame having a $\frac{3}{8}$ -inch border with a lead to the grounded point. When set down upon the crystal, the upper electrode forms a chamber or electrode water cell.

The lower surface of the upper electrode was smeared with a thin film of vaseline, and the chamber was filled with water, in which the material is exposed. Intensities and exposure periods were determined by preliminary experiments. A medium intensity, as indicated by a reading of 5 amperes in the primary circuit and 100 milliamperes in the secondary circuit, was found most satisfactory.

The water temperature in the electrode cell rose approximately 6° C. during the average exposure required, but this rise was decreased to approximately 2° C. by the introduction of a glass capillary-tube cooling system. Control experiments with a similar rise in temperature showed no appreciable effect during the $2\frac{1}{2}$ -4-minute exposures.

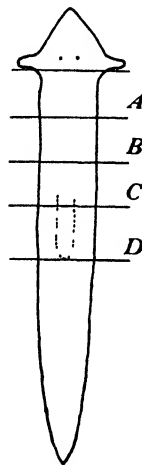


FIG. 1.-- Levels A, B, C, and D, used in all head-frequency experiments.

THE EFFECT OF SUPERSONIC VIBRATIONS ON INTACT ANIMALS

Groups of 75 and 5 worms were used in each test for determining the resistance of a large group, as compared with few individuals. The four relative intensities which were used in the scale from 4 to 7 amperes (reading in the primary inductance circuit of the oscillator) showed the lower resistance of few individuals, as contrasted with the higher

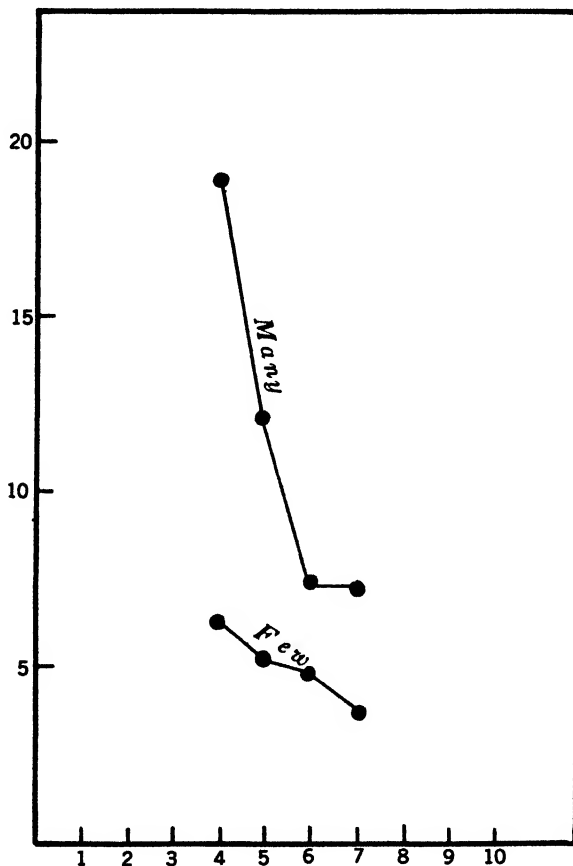


FIG. 2.—Comparison of resistance of few (5) and many (75) individuals in a group exposed to supersonic vibrations.

resistance of many individuals composing a group. These results are graphically shown in Figure 2. The median relative intensities were employed in a scale of 0-10, to gain some indication as to the trend of intensity effect upon the groups.

The course of disintegration in the individual worms when only a few were exposed showed the characteristic patterns of differential susceptibility. With prolonged exposure the pharynx is everted through the dorsal body wall.

In the lots of 75 worms, cytolysis was not uniform. In some individuals both head and posterior zooid region disintegrated, the pharyngeal region remaining intact; complete reconstitution occurred after a few days. In others, head and prepharyngeal regions

disintegrated, pharynx and posterior zooid regions were intact, and a new head regenerated. In still others, the posterior zooid regions disintegrated, the anterior zooid remained intact, and reconstitution occurred.

The average for the different experiments for each intensity showed that for a few individuals undergoing supersonic treatment there is only a slight rise in disintegration

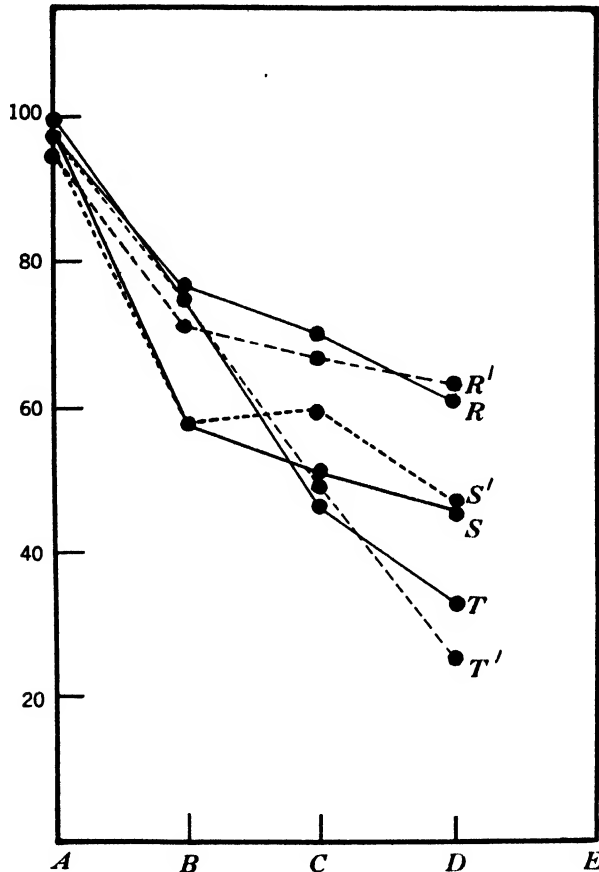


FIG. 3.—Head frequencies of pieces of worms from three different lots exposed to supersonic vibrations immediately after cutting. *R*, control; *R'*, experimental (14–16 mm.). *S*, control; *S'*, experimental (10–12 mm.). *T*, control; *T'*, experimental (12–14 mm.).

time as the intensity is decreased. With many individuals the average shows a decided protection to the group as a whole, and it is well above the level of the plotted curve for few individuals. With a decreased intensity the massed individuals show a curve that rises rapidly, which means a great increase in disintegration time.

The lots of grouped individuals tend to come together in masses, and dense secretions of mucus are formed. This is caused by the physical contact of worms and the rapid vibration of the water. Such aggregation affords protection to the individual worms in

the group. This adds another instance of group protection to the large number of such cases which have been reported by Allee and his associates (Allee, 1931, 1934). One may conclude that the following experiments with pieces in groups are affected as aggregations rather than as isolated pieces.

EFFECT OF EXPOSURE IMMEDIATELY AFTER SECTION

Experiments were first performed to determine whether supersonic treatment would affect the reconstitution of pieces cut from worms. The fourteen experiments involving

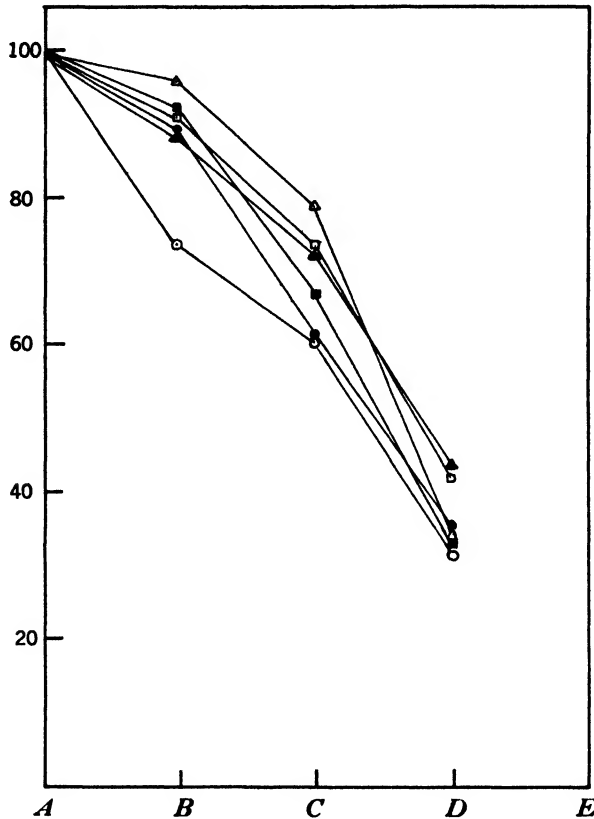


FIG. 4.—Head frequencies of 10-12-mm. worms with delayed exposure. Open circle, control; solid circle, 10 hours' delay before exposure; solid square, 18 hours' delay before exposure; solid triangle, 24 hours' delay before exposure; open square, 30 hours' delay before exposure; and open triangle, 42 hours' delay before exposure.

1,000 worms, exposed at an intensity of 5 amperes in the primary circuit and 100 milliamperes in the secondary circuit. The animals were collected at various times during the year. The supersonic treatment lasted from $2\frac{1}{2}$ to 4 minutes. The duration of the exposure varied for the different lots, depending upon the time required to cause cytolysis of ten A pieces. The exposure time that would not cause visible cytolysis was applied to pieces A, B, C, and D (approximately successive fourths of the anterior zoid).

Although the head frequencies of the pieces of the different stocks did not always coincide (Rulon, 1936), the tests are about the same as the controls, except that in the 10-12-mm. stock of *C* pieces there is an increase (Fig. 3). Experiments in which the death-rate is high often raise the question whether a change in head frequency which may appear is real or due to the death of those which would have a lower head frequency if they had lived. There is an increase in the above-mentioned *C* pieces, and it seems to

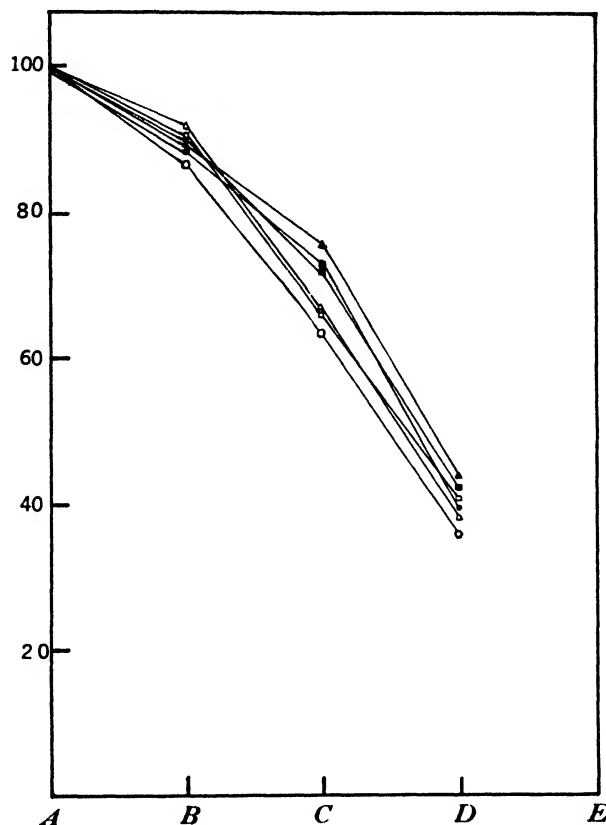


FIG. 5.—Head frequencies of 12-14-mm. worms with delayed exposure. Open circle, control; solid circle, 10 hours' delay before exposure; solid square, 18 hours' delay before exposure; solid triangle, 24 hours' delay before exposure; open square, 30 hours' delay before exposure; and open triangle, 42 hours' delay before exposure.

be real, since there are no deaths in the control and only 2.4 per cent deaths in the experimental lot. The index of the control is 50.9; that of the experimental lot, 59.9. In the 14-16-mm. pieces the slightly lower head frequency in pieces *A*, *B*, and *C* is perhaps an inhibition of the head-forming cells but is not sufficient to be significant.

DELAYED EXPOSURE

Twenty-four experiments, including 1,900 worms, were performed with various periods of delay between section and exposure. Each experiment consisted of a control and

test following 10, 18, 24, 30, and 42 hours' delay. The duration of exposure was determined as stated above. Head-frequency indexes and percentages living are tabulated in Tables 1 and 2, and Figures 4 and 5 show the increases for the two sizes of worms used in the experiments in *B*, *C*, and *D* pieces. The increase becomes greater as the period of delay increases, except that with the longest periods the index decreases in some cases.

The pieces taken from 10-12-mm. worms showed the greatest increase in *B* and *C* pieces with a 42-hour delay, and in *D* pieces with 24-hour delay (Table 2); those from

TABLE 1
DELAYED EXPOSURE: 800 WORMS, 10-12 MM.

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Control.....	99.4	73.4	60.0	31.1	92.3	99.1	98.1	94.3
10-hour delayed.....	97.9	89.1	60.2	34.8	47.5	75.0	89.5	86.7
18-hour delayed.....	100.0	91.9	66.3	31.4	27.1	87.5	70.9	78.6
24-hour delayed.....	99.1	88.6	71.9	43.4	89.1	97.1	68.3	80.6
30-hour delayed.....	99.5	90.3	73.0	41.7	75.5	89.0	92.0	72.5
42-hour delayed.....	99.3	95.3	78.6	34.4	83.8	93.0	73.5	72.1

TABLE 2
DELAYED EXPOSURE: 1,100 WORMS, 12-14 MM.

	HEAD-FREQUENCY INDEX				PERCENTAGE LIVING			
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Control.....	99.8	86.8	63.6	36.0	100.0	100.0	89.2	95.0
10-hour delayed.....	97.5	88.1	72.4	39.3	37.3	75.3	92.9	81.8
18-hour delayed.....	99.4	89.5	72.3	42.0	91.5	98.6	97.4	100.0
24-hour delayed.....	98.8	88.6	75.4	43.5	75.2	89.7	91.6	89.7
30-hour delayed.....	100.0	89.7	66.4	40.6	85.3	86.2	96.6	97.5
42-hour delayed.....	100.0	91.1	66.7	37.7	97.5	97.9	81.4	81.6

12-14-mm. worms in *C* and *D* pieces with 24-hour delay, in *B* pieces with 42 hours' delay. The tables show that in a few of the lots the death-rate is high, and these are therefore open to suspicion. However, the increase occurs in the lots with few or no deaths as well as in those with high death-rates.

CONCLUSION

The increase in effect on head frequency with delayed exposure probably means that, after exposure immediately following section, recovery occurs so rapidly that head frequency is only slightly or not at all affected. The apparent decrease of the head-frequency index with the longer period of delay may mean that, as regeneration of the head progresses, the cells giving rise to the head become more susceptible. The inhibiting effect on the physiological state of these cells begins to appear in head development.

The factor which inhibits head development in short posterior pieces is apparently

a stimulation resulting from the posterior cut, and at least in considerable part it is nervous in character (Watanabe, 1935). The observed increase in head frequency presumably results from an inhibition or decrease of this stimulation, but how supersonic vibrations determine this effect is not known. As regards head frequency, supersonic waves act essentially like other inhibiting agents. Their effect on pieces with low head frequency is manifested to a greater degree on the factor inhibiting head development than on the head-forming cells. Further experiments may more definitely indicate the manner in which the effects of supersonics on organisms are produced.

SUMMARY

1. With a certain range of intensity and duration of exposure, supersonics bring about cytolysis in whole individuals of *E. dorotocephala*. This cytolysis progresses from the anterior end posteriorly in the anterior zoid, just as is the case when the worms are exposed to certain other physical and chemical agents.

2. On return to water after partial cytolysis, reconstitution occurs.

3. Worms exposed in groups of few individuals are more susceptible than in groups of many individuals.

4. In general the effect increases with intensity and duration of exposure, but individual differences occur as with other agents.

5. For determination of the effect on head frequency, experimental and control lots of pieces *A*, *B*, *C*, and *D* (approximately successive fourths of the anterior zoid) were used, since these pieces give progressive decrease and a wide range of head frequency from *A* to *D*. Effective intensities and exposure times were determined by preliminary experiment. Lots were exposed immediately after section and following various periods of delay.

6. With exposure immediately after section little change in head frequency occurred.

7. With delayed exposure an increase in head frequency occurs and is apparently greater with certain periods of delay than with longer or shorter periods.

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TESTS FOR SEXUAL DIFFERENTIATION IN *PARAMECIUM MULTIMICRONUCLEATUM* AND *PARAMECIUM CAUDATUM*

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CONJUGATION of a number of races of *Paramecium multimicronucleatum* Powers and Mitchell has been found to be correlated with environmental changes (Giese, 1935 and 1938). Sonneborn (1937) has found that for induction of conjugation in certain races of *P. aurelia* Müller two strains, which he calls "sexes," are necessary.

This paper summarizes attempts to determine (1) if sexual differentiation is present in any or all of the strains of *P. multimicronucleatum* and *P. caudatum* Ehrenberg in use in this laboratory and (2), if so, what effect, if any, environmental changes play when such sexual differentiation is present.

EXPERIMENTAL

Races 2E, 2C, 6, 7, 8, 9, 11, and 13 (most of these previously described [Giese, 1938]) of *P. multimicronucleatum* and races 4, 12A, 12B, and 14 of *P. caudatum* were used in this study. Stock cultures were maintained in the manner previously described (Giese and Taylor, 1935). Isolation cultures were grown in small tubes, 7 cm. long and 4 mm. in inner diameter, in the same medium and with the same precautions as for the larger cultures. Temporary slides were made with acetocarmine; permanent mounts were made with Schaudinn's fixative and the Feulgen reaction.

In a race showing genotypic sexual differentiation, progeny of a single individual should not conjugate without the reorganization of nuclear apparatus such as occurs during endomixis. If the progeny of a single individual can conjugate among themselves, either sexual differentiation in the rigid sense is absent or sex reversal among some of the members of a given genotype must be induced before conjugation can occur. Single animals of a race to be tested for sex were isolated into small tubes; and, after a number of divisions had occurred, one vigorous culture was chosen as stock. From this stock one individual was inoculated into each of a set of five to fifteen tubes. This was repeated from five to fourteen times for each of the various races tested. Stranghöner (1932) found that complete reorganization after endomixis may take as long as ten generations in *P. multimicronucleatum*. To determine whether endomixis was occurring in our cultures, samples were stained daily in some cases, at the time of transfer (about every 48 hours in most cases) in others. Examinations for conjugation were made every 12 hours; and in case no conjugants were observed in a given culture, the examinations were continued for 6 days after subculture, at which time the cultures were abandoned, as the animals were minute and not likely to conjugate.

Growth occurred rapidly in the small tubes, and the data on division-rate for three races of *P. multimicronucleatum* and for two races of *P. caudatum* are given in Table 1. In Table 2 are summarized data on tests for sex.

Examination of Table 2 shows that progeny of a single individual conjugate without intervention of endomixis in all of the races of *P. multimicronucleatum* except 2C but

TABLE 1
DIVISION-RATES OF A NUMBER OF RACES OF *Paramecium*

Species	Race Number	Time (in Hours) after Inoculation	Trials	Average Divisions per Day
<i>Paramecium multimicronucleatum</i>	2C	24	171	2.5
		48	91	2.3
		72	79	2.0
		96	32	1.6
	6	24	92	2.9
		48	47	2.8
		72	39	2.4
	9	24	79	2.6
		48	25	2.5
		72	47	2.4
	12A	24	48	3.1
		48	23	2.6
		72	20	2.4
<i>P. caudatum</i>	12B	24	50	3.1
		48	25	2.7
		72	26	2.5

TABLE 2
CONJUGATION AMONG PROGENY OF A SINGLE VEGETATIVE INDIVIDUAL
DIVIDING WITHOUT INTERVENTION OF ENDOMIXIS

Species	Race Number	Number of Series Studied	Total Number of Trials	Percentage of Total Trials Showing Conjugation
<i>Paramecium multimicronucleatum</i>	2C	5	60	0
	2C	7	21	0
	2E	5	25	100
	6	14	260	(50)*
	7	12	85	100
	8	5	40	74
	9	6	51	100
	9	12	228	(75)*
	11	5	66	87
	13	5	25	25†
<i>P. caudatum</i>	4	5	25	0
	14	7	28	0
	12A	7	35	0
	12B	7	35	0

* In percentage of the series; data not kept for each trial. Both of these series were started from a single exconjugant.

† Cultures of race 13 were never very healthy and multiplication was always slow.

not in any of the races of *P. caudatum*. When conjugation occurred, it began, at the earliest, 72 hours after subculture. Individuals of races 8, 11, and 13 divided more slowly than the others, and in such cases conjugation was correspondingly delayed.

Progeny of a single exconjugant of race 6 were used in the experiments reported here on this race. After a period of very rapid multiplication, the division-rate dropped quickly, until finally, even after transfer to fresh medium, there were fewer than 0.5 divisions per day and experimentation was abandoned. Fourteen series were studied in all; in the first five, no conjugation occurred; thereafter conjugation occurred in seven of the nine remaining series. Stained preparations, either temporary or permanent, were made daily. No endomixis was observed to occur. However, macronuclear breakdown of the type described as "hemixis A" by Diller (1936), unaccompanied by micronuclear activity and macronuclear reorganization, was observed to occur. This macronuclear breakdown was found only among some of the offspring of a given individual. Similar but less marked macronuclear fragmentation was observed in occasional members of races 2 and 11 but not in any of the other races studied.

One of the cultures developed from isolated individuals of race 2 and its subcultures consistently showed conjugants without intervention of endomixis; others did not. The conjugating clone (labeled 2E) and one of the nonconjugating clones (labeled 2C) were isolated for further study. When in a series of six experiments 50 members of each race were mixed together and kept at about 20° C., 84 per cent of the animals in the mixed cultures conjugated; whereas, in controls (100 individuals) of 2E alone, 64 per cent of the animals conjugated; and in controls of race 2C alone, no conjugants were observed to occur. Whereas they would not conjugate among themselves, individuals of race 2C were conjugating with individuals of race 2E. When a single individual of 2E was placed in a culture of 2C, one and only one pair of conjugants was obtained. When 5 individuals of 2E were placed in a culture of 2C, never more than 5 conjugants were obtained (for five experiments: 3, 4, 5, 5, 5). Individuals of 2C cannot conjugate with one another but will conjugate with individuals of 2E; to this extent we may speak of sexual differentiation in this race.

If 2E and 2C are to be considered as opposite sexes, one might expect one or the other of them to mate with individuals of the other races of the same species. In a number of trials mixtures were made of individuals of races 7, 8, 9, and 11 with 2C, and also with 2E, at the time when each of the races tested was in a state of incipient conjugation (sticky state). Individuals of race 2 (E or C) can be distinguished from the individuals of the other races by the fact that the excretion crystals are much larger and usually much more numerous than in the other races. In no case were conjugants obtained in which a member of race 2 was conjugating with a member of any of the other races. This either indicates incompatibility or inadequate control of environment, although an attempt was made to mix only animals in incipient conjugation. Conjugants occurred in the mixtures, but examination showed members of pairs to be of the same race. Conjugants were obtained also in the unmixed controls except in cultures of race 2C.

Individuals of race 2E show a peculiar type of sexual "segregation" following vegetative divisions. For, if one isolates a pair of individuals in incipient conjugation, then separates them, and grows a culture from each individual, mixtures of the two cultures (50 individuals of each race were mixed; controls consisted of 100 of the unmixed individuals of each race) show a higher percentage of conjugation than does each culture separately under the same environmental conditions. Data for the first series are sum-

marized in Table 3. Similar tests (four trials) were made upon cultures obtained by isolating each of the individuals of two pairs in incipient conjugation in race 2Eg (Table 3) of the first series. Another series of tests (five trials) were made upon two similar pairs from one of the races of the second series (race 2Eg₁) and a final set of similar trials with one of the pairs of incipient conjugants obtained from 2Eg₁. The data from these series are omitted for want of space, but they are comparable to the data in Table 3 for the first series. As indicated in the data in Table 3 for mixtures of members of different pairs and for similar mixtures in other series, members of the different pairs are like or unlike one another. The data as a whole suggest correlation of "sex" not with

TABLE 3

PERCENTAGE IN CONJUGATION IN MIXED AND UNMIXED CULTURES DERIVED (WITHOUT INTERVENING ENDOMIXIS) FROM EACH OF THE INDIVIDUALS OF INCIPIENT PAIRS OF CONJUGANTS OF RACE 2E OF *Paramecium multimicronucleatum*

TRIAL	PAIRS									MIXTURE OF MEMBERS OF DIFFERENT PAIRS			
	1			2			3*	4*	5*				
	a†	b	a×b	c	d	c×d	e	f	g	af	ag	bg	ef
1.....	o	o	70	28	o	78	o	o	o	o	36	58
2.....	o	26	50	20	4	84	4	o	4	o	78	92
3.....	o	o	82	o	26	96	o	o	8	o	68	26
4.....	o	o	80	o	50	22	o	o	o	60	o	70	o
5.....	o	4	58	o	10	6	o	o	2	18	o	66	2
6.....	o	o	64	o	18	70	10	16	8	64	4	56	76
7.....	o	8	94	2	14	92	20	o	16	82	o	80	84
8.....	24	4	50	2	4	60	20	6	2	10	66†	78	20

* The other member of each of these pairs failed to give rise to a healthy culture.

† Reason for sudden change not known.

‡ The letters under each of the numbers refer to the members of a pair.

the macronucleus but with something less regularly distributed in vegetative division, possibly the micronucleus, since Stranghöner (1932) found such irregular distribution of the micronuclei in division.

In none of the series of trials with the four races of *P. caudatum* was conjugation ever observed to occur in cultures descended from a single individual without the intervention of endomixis.¹ Tests and observations were made in the same manner as for the other species. However, when two of the races, labeled 12A and 12B, were mixed at the right state in development or grown together, conjugants were obtained in large numbers. A large number of tests with 15-cc. cultures indicated that conjugation was never obtained unless the two strains were present. This is like the situation described by Sonneborn (1937) for some races of *P. aurelia*.

Individuals of race 4, however, when mixed with either of the strains of race 12, did not conjugate. Similar tests with race 14 showed no conjugants, as did also mixtures of

¹ After endomixis in race 12B sexual differentiation occurred and conjugants were obtained.

4 and 14. To make sure that the proper period for conjugation was not missed, experiments were tried at various times after subculture; and, also, animals of each of these races were grown together: 4 with 12A, 4 with 12B, 4 with 14, 14 with 12A, and 14 with 12B. The incompatibility of the sexes of race 12 with races 4 and 14 is at present incomprehensible. It is possible that some peculiar environmental condition necessary for conjugation was not supplied.

Where there is no clear-cut differentiation into sexes, as in races 6, 7, 8, 9, 11, and 13, the onset of conjugation is correlated with a definite environmental change (Giese, 1935, 1938), conjugation occurring only when there is a sudden decline in the nutritive conditions after a period of plenty. It is of great interest to determine if for the races showing sex differentiation (2E and 2C of *P. multimicronucleatum*, and 12A and 12B of *P. caudatum*) this is true, or whether, as in one of the races of *P. aurelia* described by Sonneborn (1937), individuals full of food will conjugate. To this end the following experiments were performed.

Individuals of race 2E, when still full of food, were added to similar individuals of race 2C. No immediate reaction occurred, and it was only after starvation that conjugants were obtained. Conjugants occurred for a period of 3 days; when pairs appeared, they were removed. It was demonstrated that 84 per cent of the animals had conjugated; but the epidemic was prolonged, the animals pairing only when they had reached a certain physiological state.

If both sets of paramecia had been starved subsequent to feeding, the clumping was immediate upon mixture, and within 2 hours the majority had fused in pairs; a count after 5 hours indicated that 88 per cent had paired. Such experiments were repeated many times with the same general result.

Results indicating similarly the importance of the environment were obtained in another series of experiments in which animals of 2E were introduced into cultures of 2C, starved to a point where they would quickly unite with properly starved members of race 2E. If 1 individual of 2E is introduced, a single clump of sometimes 10 or more members of 2C form about this animal. When the successful animal of 2C begins to pair with 1 individual of 2E, the others cease clumping about the pair. If one introduces 5 animals of 2E into a culture of 2C, generally 5 clumps are formed, never more than 5. If conjugants of 2E \times 2C or 2E \times 2E are introduced, there is no clumping. If dividing individuals of 2E are introduced, there is no clumping. If an individual of 2E, in the proper physiological state for conjugation but somewhat injured (large vacuole appearing within), is introduced, clumping occurs; but if the individual is killed by heat or by ultra-violet light, it no longer attracts the individuals of 2C. If an individual of 2E in the proper state for conjugation is placed into a suspension of the food bacterium and then, after the formation of food vacuoles, is quickly rinsed and placed with individuals of 2C, clumping occurs, followed by the usual pairing-up. Apparently, while the well-fed individual must be starved to achieve a certain state before it can conjugate, a mere stuffing with bacteria does not change the physiological state conducive to conjugation. A certain period must elapse after the food is taken in to change the physiological state. If too long a time elapses after feeding, the "attractive state" is lost, for a well-starved animal of 2E no longer causes clumping in a culture of 2C. The clumping observed in the experiments suggests some diffusible attractive substance; but, so far, attempts to isolate any substance in agar or in other ways have failed.

The results clearly indicate that, when sex is present, and at least members of race 2C

must be mixed with 2E to obtain conjugation, both races (sexes) must be in the proper physiological state and in the proper environment before they will unite in conjugation.

Very similar results were obtained with *P. caudatum*. When well-fed individuals of strains 12A and 12B were mixed, no conjugants were observed until depletion of food was evident. When the animals were in the proper physiological state for conjugation, i.e., starved after a period of plenty, clumping occurred immediately after mixing, and pairing-up followed within 2-5 hours. If well-fed individuals of 12A were mixed with properly starved individuals of 12B, conjugants were not obtained; and the reciprocal experiment gave similar results. If either strain was starved too long, even if the other were in the proper physiological state for conjugation (as determined by mixing with the opposite sex in the proper physiological state and obtaining conjugants), conjugation failed to occur; and if both strains were starved too long, similar failure to conjugate was observed.

Environmental changes alone fail to induce conjugation when only one sex is present (cf. studies on race 2 reported in Giese, 1938), but environment may condition the physiological state conducive to conjugation even when sexual differentiation is present. It is also possible that certain environmental conditions may favor or suppress sex reversal in a given genotype. A knowledge of the effect of variations in the environment upon conjugation is therefore important for an understanding of the nature of the factors inducing conjugation.

SUMMARY

1. The majority of the races of *P. multimicronucleatum* tested showed conjugants even without the intervention of endomixis. In one race distinct separation into "sexes" was found.
2. Of four races of *P. caudatum*, two were found to be of opposite "sexes," conjugating only on mixture; the other two were not observed to conjugate with each other or, when mixed, with either of the other two strains.
3. In races in which conjugation occurred among progeny of a single individual without intervention of endomixis, the pairing followed exhaustion of food after a period of plenty.
4. Correlation of onset of conjugation and the exhaustion of food was also observed in the races showing separation into "sexes," for mere mixture when the animals were well nourished did not result in conjugation until the food supply was relatively depleted.

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THE QUESTION OF AUTOTROPHIC NUTRITION IN *EUGLENA GRACILIS*

(Three figures)

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THE trophic nature of *Euglena gracilis* has been investigated from time to time over a period of nearly forty years and is still a disputed question. Zumstein (1899) and Ternetz (1912) concluded that inorganic media are unfavorable to growth of *E. gracilis*. Pringsheim (1912), on the other hand, reported that this species grew almost as well in certain inorganic solutions as in media containing organic food, and Mainx (1928) likewise concluded that the species is facultatively autotrophic. Dusi (1933a), moreover, carried the Mainx strain through several successive transfers in inorganic media. More recently, Hutner (1936) has been unable to grow *E. gracilis* in such media, whereas Dusi (1937) has reaffirmed his earlier statements.

The need for a chlorophyll-bearing species which could be used as an autotrophic control in certain investigations on nutrition of colorless Euglenida led the writers to attempt the growth of *E. gracilis* in inorganic media. The failure of our first few attempts afforded no explanation for the contradictory results of Dusi and Hutner and has necessitated a more extensive investigation with several different inorganic media.

A bacteria-free strain of *E. gracilis*, obtained in 1931 from Professor E. G. Pringsheim, has since been maintained in pure culture in our laboratory. This strain was the source of the flagellates used in the present investigation. Satisfactory media for stock cultures have been described elsewhere (Hall, 1937a). The media used in the experiments on autotrophic nutrition are listed on page 77. When necessary, each medium was adjusted so that the pH after sterilization would fall within the optimal range (pH 6.5-7.5) for *E. gracilis* (Jahn, 1931; Dusi, 1933a). The solution was then tubed in measured amounts and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

In the first transfer of each experimental series the culture tubes were inoculated from a stock flask or tube containing the appropriate inorganic medium and previously seeded from a stock culture of *E. gracilis*. In two series, specified below, the stock flask was inoculated from an agar slant culture by means of a platinum loop, so that very little organic material was carried over from the original culture. In other series the stock flask was inoculated by means of a graduated pipette from a culture in peptone medium. Each tube of the first transfer received a 0.5-cc. or, in certain cases, a 1.0-cc. inoculum from the stock flask. In each transfer approximately fifteen tubes were inoculated. Four of these were fixed for the initial count (number of flagellates per cubic centimeter), one was used for determination of the initial pH, and the remainder were incubated at room temperature near a window. After incubation four tubes were fixed for the final count, one was used for determination of final pH, and another for inoculation of the second transfer. The third transfer, in turn, was inoculated from a tube of the second transfer; and the same procedure was followed in subsequent transfers.

All pH determinations were made with a La Motte roulette colorimeter. Initial and

final counts were made with a Whipple ocular micrometer and a Sedgwick-Rafter counting cell, as described previously (Hall, Johnson, and Loefer, 1935). In the calculation of peptone concentrations (grams per cubic centimeter) in successive transfers, the utilization of peptone by the flagellates was not considered. Hence, the concentrations listed below are conservative estimates of the peptone present.

Medium D ("solution D" of Mast and Pace, 1933):		Medium EC (Mainx, 1928; Dusi, 1933b):	
NH ₄ Cl.....	0.497 gm.	(NH ₄) ₂ HPO ₄	1.0 gm.
MgSO ₄ (anhydrous).....	0.048 gm.	K ₂ HPO ₄	0.2 gm.
K ₂ HPO ₄	0.209 gm.	MgSO ₄ · 7H ₂ O.....	0.1 gm.
Sodium acetate.....	1.148 gm.	FeCl ₃	0.0025 gm.
Distilled water.....	1.0 liter	Distilled water.....	1.0 liter
Medium EA (used by Loefer, 1934, for <i>Chlorogonium</i>):		Medium EF:	
NH ₄ NO ₃	0.5 gm.	NH ₄ NO ₃	1.0 gm.
KH ₂ PO ₄	0.5 gm.	MgSO ₄ · 7H ₂ O.....	0.2 gm.
MgSO ₄ · 7H ₂ O.....	0.1 gm.	CaCl ₂	0.1 gm.
NaCl.....	0.1 gm.	KH ₂ PO ₄	0.2 gm.
FeCl ₃	0.0025 gm.	FeCl ₃	0.0025 gm.
Distilled water.....	1.0 liter	Distilled water.....	1.0 liter
Medium EAB: same formula as medium EA but contains, in addition, MnCl ₂ · 4H ₂ O in a concentration of 5×10^{-7} molar.		In addition, this medium contained MnCl ₂ · 4H ₂ O in a concentration of 5×10^{-7} molar.	

GROWTH OF *Euglena gracilis* IN INORGANIC MEDIA

Series I and II (medium D).—In these preliminary series a stock flask seeded from an agar slant culture was used for inoculating tubes of the first transfer. The peptone concentration in the culture tubes was thus reduced to 2.1×10^{-7} in series I and 3.6×10^{-7} in series II. The initial pH was 7.0 in each case, and the initial counts were 683 per cubic centimeter in series I and 340 in series II. After 17 and 19 days of incubation, respectively, x/x_0 (ratio of final to initial count) was 18.4 in series I and 15.0 in series II, indicating moderate growth in each case. The second transfers were incubated for 17 days, when no growth was noted in either series. Since the calculated peptone concentrations were 1.3×10^{-8} in series I and 2.5×10^{-8} in series II, it was decided to avoid such an abrupt change from organic to inorganic media in subsequent experiments.

Series III (medium D).—Initial pH was 6.5 in the first transfer and 6.9 in the second and third. Initial counts were: 4,893, 3,542, and 280, respectively. Incubation periods were 14 days in the first transfer and 21 days in the second and third. Calculated peptone concentrations were 5×10^{-5} , 2.5×10^{-6} , and 1.2×10^{-7} in successive transfers. The results (Table 1) showed moderate growth in the first transfer and none in the second and third. The series was discontinued after the third transfer.

Series IV (medium D).—The initial pH was 6.8, 6.9, and 6.8 in successive transfers; and initial counts were: 14,733, 1,087, and 147. Calculated peptone dilutions were the same as in series III. Incubation periods were 13, 21, and 21 days. In the first transfer (Table 1) growth was comparable to that in the first transfer of series III; in the second, the apparent increase in number was not statistically significant; and in the third trans-

fer there was a definite decrease in number of the flagellates. The results obtained in series I-IV thus indicate that medium D will not support growth of *E. gracilis* through successive transfers.

Series V (medium EA).—The initial pH was 6.6, 6.8, 6.9, and 6.8 in successive transfers; and initial counts were: 1,653, 8,075, 587, and 106. Calculated peptone dilutions were 5.2×10^{-5} , 3.3×10^{-6} , 1.6×10^{-7} , and 8×10^{-9} . Incubation periods were 8, 12, 9, and 22 days, respectively. Good growth (Table 1) occurred in the first transfer, poor growth in the second, and a decrease in number of flagellates was evident in both the third and fourth.

TABLE 1

SERIES III-IX: GROWTH OF *Euglena gracilis* IN INORGANIC MEDIA

Growth in the successive transfers of each series is expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter).

SERIES	x/x_0 IN SUCCESSIVE TRANSFERS, DIFFERENT SERIES			
	First	Second	Third	Fourth
III.....	6.5	1 0	0.8
IV.....	6.8	1 3	0.5
V.....	45.7	2.6	0.4	0.3
VI.....	41.4	2.8	1.5	62 4
VII.....	18.6	1.9	8.7
VIII.....	2 3	1 1	1.2	0.6
IX.....	13.5	1 0	13.7	1.0

Series VI (medium EC).—The initial pH was 7.0, 7.0, 6.9, and 7.0 in successive transfers; and the initial counts were: 680, 3,190, 620, and 53. Calculated peptone concentrations were: 4.5×10^{-5} , 2.2×10^{-6} , 1.1×10^{-7} , and 5.5×10^{-9} , respectively. Incubation periods were 8, 12, 21, and 18 days. The results (Table 1) showed good growth in the first and fourth transfers and slight increase in number of flagellates in the second and third transfers. Unfortunately, the departure of the junior author made it necessary for us to discontinue this series in the fourth transfer. The encouraging results led us to try the same medium in later experiments, one of which proved successful.

Series VII.—In this case ammonium phosphate was omitted from medium EC. Calculated peptone concentrations were approximately the same as for the first three transfers in series VI. Initial counts were 551, 633, and 93. Initial pH was 7.0, 6.9, and 7.0; and the incubation periods were 11, 12, and 21 days, respectively. Growth was apparent in each transfer, although much less in the second than in the other two. The results indicated that growth in the first three transfers may be attributed to the peptone carried over from the original stock culture.

Series VIII (medium EC).—Initial pH was 7.0, 7.1, 6.9, and 7.1 in successive transfers; and initial counts were 2,245, 3,900, 153, and 360. Calculated peptone concentrations ranged from 5×10^{-5} in the first transfer to 6×10^{-9} in the fourth. In inoculation of the fourth transfer, concentrated material from one tube of the third transfer was inoculated into another tube, and the latter was used for inoculation of the fourth transfer. In this way the initial count was increased to 360. The transfers were incubated for 17, 18, 20, and 21 days, respectively. The results (Table 1) showed poor growth in

the first transfer, none in the second and third, and a decrease in number of flagellates in the fourth. The conflicting results of series VI and VIII were puzzling until it was discovered that different preparations of ammonium phosphate had been used in the two series. In a later series (XII), medium EC was made up with the same type of salt as that used in series VI, and the results showed better agreement with those of the latter series.

Series IX (medium EAB).—The addition of manganese to an inorganic medium was suggested by earlier observations (Hall, 1937b) that growth of *E. anabaena* is accelerated by this substance in certain concentrations. In this series initial pH was 7.1, 6.9, 6.9, and 6.7 in successive transfers; and initial counts were: 2,245, 3,900, 313, and 276. The transfers were incubated for 8, 16, 21, and 20 days, respectively. Calculated peptone concentrations were the same as in series VIII. The results (Table 1) showed growth in the first transfer, none in the second, growth in the third, and none in the fourth. Because of the low concentration of flagellates in the fourth transfer, the series was discontinued.

At this point a series of viability tests was carried out on old cultures at various transfers in media EA and EAB. At intervals tubes of peptone medium were inoculated in 1.0-cc. amounts from the cultures in inorganic medium and then incubated at room temperature. The results showed that *E. gracilis* was still viable in medium EA after 49 days in the first transfer, 36 days in the second, 19 days in the third, and 30 days in the fourth transfer. No attempt was made to determine the maximum in any case. The results for medium EAB showed survival for 30 days in the first transfer, 14 days in the second, and 26 days in the third. Obviously, neither of these media was lethal, since the flagellates survived at least in small numbers even in those transfers which showed no growth. These results led us to concentrate the flagellates before inoculating the third and fourth transfers in series X, in the hope of carrying over enough viable flagellates to maintain growth in the transfers.

Series X (medium EAB).—In different transfers the initial pH ranged from 6.7–7.0. Initial counts in the first twelve transfers were as follows: 2,307, 5,460, 700, 217, 1,573, 987, 360, 233, 900, 520, 553, and 813. Incubation periods were 14 days in the first, 20 in the second, and 21 days in subsequent transfers. The calculated peptone concentration was reduced from 10^{-4} in the first transfer to 3.5×10^{-19} in the twelfth transfer. Before inoculation of the third transfer, 1.0 cc. of concentrated material from one second-transfer tube was added to another, and the latter was used as stock for the third transfer. For the fourth transfer a similar procedure was followed. This modification of technique was introduced for the purpose of increasing the size of the initial count. The results (Fig. 1) showed good growth in the first transfer, poor growth in the second, none in the third, excellent growth in the fourth, and fairly good growth in all succeeding transfers. The series is now in the fifteenth transfer, with no marked change in growth-rate.

The first four transfers in this series proved to be interesting. In the second and third transfers it was noted that the majority of organisms were etiolated and otherwise abnormal in appearance, while a few were comparatively large, densely green, and quite active. In the fourth transfer the change was striking. Except for rare specimens, all of the flagellates were bright green and healthy in appearance. This type has been predominant in all the later transfers. The same phenomenon had been observed previously in series VI (Table 1), a series which was discontinued in the fourth transfer. These

observations suggest a selective action of inorganic media in which many of the flagellates die in the first few transfers. The survivors, on the other hand, appear to be capable of continued growth in such media.

Series XI (medium EF).—The initial pH ranged from 6.6 to 7.5 in different transfers; and the initial counts in the first twelve transfers were: 2,147, 1,407, 147, 1,067, 567,

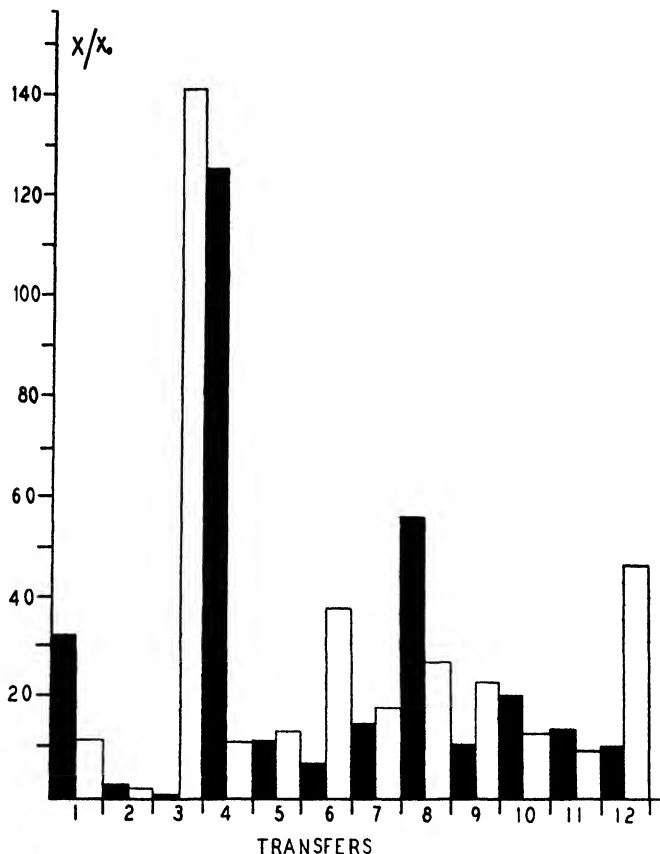


FIG. 1.—Series X and XI. Growth in successive transfers is expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter); series X in black, series XI in white blocks.

233, 540, 387, 513, 647, 340, and 320. The incubation period was 21 days in each case. Moderate growth was noted in the first transfer, very little in the second, excellent growth in the third, and moderate growth in subsequent transfers (Fig. 1). The calculated peptone concentration was reduced from 10^{-4} in the first transfer to 5×10^{-19} in the twelfth. Medium EF thus appears to be adequate for autotrophic nutrition of *E. gracilis*.

This series was similar to series X, except that the recovery in growth-rate occurred in the third transfer instead of the fourth. The same contrast in appearance of the two types of flagellates was noted in the second transfer of series XI, and practically all of

the flagellates observed in the third transfer were normal in appearance. It was decided at this time to start several side series from series X. It seemed probable that, if a real selection of autotrophic forms had occurred, such flagellates would continue to grow moderately well in other inorganic media. Accordingly, series XII, XIII, and XIV were inoculated each from a fourth transfer tube of series X.

Series XII (medium EC).—The initial pH ranged from 6.9 to 7.2 in different transfers; and initial counts in successive transfers were: 1,427, 487, 427, 460, 400, 853, 347, and 240. In the eighth transfer the calculated peptone concentration had been reduced to 4.5×10^{-19} grams per cubic centimeter. Each transfer was incubated for 21 days.

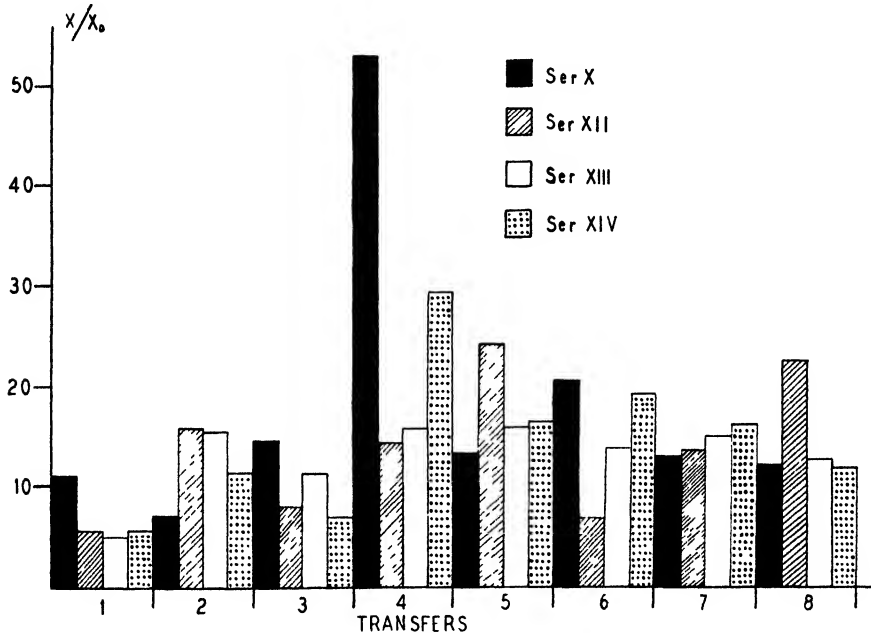


FIG. 2.—Series X (transfers 5–12, inclusive), and series XII–XIV (transfers 1–8, inclusive). Growth in successive transfers is expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter).

In the first transfer, run concurrently with the fifth transfer of series X, growth (Fig. 2) was about half as great as in the latter. In subsequent transfers, however, growth was sometimes greater and sometimes less than in the parent series. There was no indication of any selective effect.

Series XIII (medium EA).—This series was carried simultaneously with the fifth and later transfers of series X. Initial pH varied from 6.7 to 6.9; and successive initial counts were: 2,180, 987, 760, 613, 607, 880, 380, and 360. Calculated peptone concentrations were the same as in series XII, and the incubation period was also 21 days for each transfer. Growth in the first transfer (Fig. 2) was approximately the same as that in series XII, while growth in the succeeding transfers was usually comparable to that in the concurrent transfers of series X. In this series also there was no indication of any selection.

Series XIV (medium EF).—This series was started a week later than series XII and XIII. Initial pH ranged from 6.5 to 7.3; and initial counts were: 1,393, 960, 513, 307, 493, 647, 747, and 900. Each transfer was incubated for 21 days. Calculated peptone concentrations were approximately the same as in series XII. Growth in the first transfer (Fig. 2) was almost identical with that observed in series XII and XIII. Growth in subsequent transfers was fairly comparable to that in corresponding transfers of series X.

The results obtained in series XII–XIV are in accord with the assumption that a definite selection of flagellates capable of autotrophic nutrition occurred in the fourth transfer of series X.

Series XV.—This series was undertaken in an attempt to reconcile the findings of Hutner (1936) with those of Dusi (1933a) and the writers. Our medium EF, Hutner's (1936, p. 94) concentrated medium, and Hutner's diluted medium were compared in series inoculated from the same culture of one of our autotrophic strains in medium EF. All three sets of cultures were incubated under the same conditions at room temperature near a north window. In the first transfer the initial pH was 6.7 in each case; in the second transfer, 6.7 for medium EF and 6.8 for both of Hutner's media. Both transfers were incubated for 21 days. Growth (x/x_0) in the three types of media is described in Figure 3. It is obvious that our autotrophic strain of *E. gracilis* grew in both the first and second transfers in medium EF, while no growth occurred in the second transfer in either of Hutner's media and no normal green organisms were observed on microscopic examination of several samples. It thus appears that Hutner's media are much less satisfactory for growth of our autotrophic strains of *E. gracilis* than are the media used in series X–XIV.

Viability tests, as described above for media EA and EAB, were carried out on first and second transfer cultures in Hutner's media. In the case of Hutner's concentrated medium, growth was observed after 3 weeks in two out of three peptone cultures inoculated from first transfer tubes. However, no growth was obtained in peptone medium inoculated from second transfer tubes, and microscopic examination of centrifuged material showed no normal flagellates. Similar tests showed that *E. gracilis* remained viable for 7 weeks in first-transfer tubes of Hutner's dilute medium and for at least 3 weeks in the second-transfer tubes. Hutner's concentrated medium thus appears to be toxic to our autotrophic strains of *E. gracilis*, while his dilute medium still contains viable flagellates after several weeks, even though no population growth occurs.

DISCUSSION

The results described above are in agreement with the conclusions of Pringsheim, Mainx, and Dusi that *E. gracilis* is capable of carrying on photoautotrophic nutrition. Our failures in certain cases, and those of Hutner (1936), emphasize the difficulties of growing Euglenidae in inorganic media, in contrast to the comparative ease with which such a phytomonad as *Chlorogonium euchlorum* (Loefer, 1934; Hall and Schoenborn, 1938) may be established in such cultures.

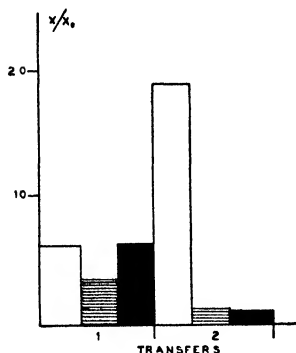


FIG. 3.—Growth of an autotrophic strain of *E. gracilis* for two transfers in medium EF (white blocks), Hutner's diluted medium (crosshatched blocks), and Hutner's concentrated medium (black blocks).

After eight unsuccessful attempts the writers succeeded in establishing two autotrophic strains of *E. gracilis*. The conditions were somewhat interesting. Viability tests had shown that the flagellates remain alive for several weeks in certain media which apparently failed to support growth after the second or third transfer. In the hope of increasing the number of viable organisms in the inoculum, the flagellates were concentrated before inoculation of the third and fourth transfers in series X. Microscopic observations showed that in the second and third transfers a few of the organisms remained bright green and healthy, while the remainder were etiolated and otherwise abnormal. In the fourth and subsequent transfers the green type has been observed almost exclusively, and fair to excellent growth has occurred. In series XI, in which no concentration of flagellates was attempted, the same phenomenon was observed, with the exception that the green autotrophic population was established in the third transfer instead of the fourth.

These observations suggest the occurrence of a selective process in which those flagellates capable of autotrophic nutrition survived, whereas the rest died in the first few transfers. It may be assumed that our pure line of *E. gracilis*, which had been maintained in our own laboratory for more than 6 years in peptone media, contained a certain proportion of flagellates capable of carrying on photoautotrophic nutrition. Upon transfer to an inorganic medium these flagellates continued to multiply, whereas the remainder were unable to become adapted to the new conditions. Such an assumption may explain the rapid decrease in rate of population growth observed in the first few transfers of series X and XI.

If such a selective process had occurred, it might follow that the resulting autotrophic strain should be able to grow in other satisfactory inorganic media without any marked change in growth-rate. This hypothesis was tested by starting several series from the fourth transfer of series X in media EC, EA, and EF. These new series (XII–XIV) showed fair to moderate growth from the first transfer on, and there were no microscopic indications of selection. While the several series showed variations in growth in different transfers, such results were to be expected, since initial pH and initial counts varied and external conditions (light and temperature) were not constant throughout the period of observation. These preliminary observations thus suggest the occurrence, in certain inorganic media, of a definite selection which eliminated many of the flagellates in early transfers and permitted survival of a strain of *E. gracilis* capable of continued growth under conditions of photoautotrophic nutrition.

The occurrence of such a process may account for some of the contradictory results obtained in studies on autotrophic nutrition of Euglenidae. The combination of a process of selection and the progressive decrease in concentration of flagellates inherent in the technique of serial transfers would greatly increase the difficulties in such investigations. The writers were fortunate in obtaining positive results in two out of ten series started from stock cultures in peptone media. Hutner (1936) did not depend upon microscopic observations for measurement of growth, and thus may have overlooked significant changes such as those observed in the early transfers of our series X and XI. In addition, the results obtained in series XV indicate that Hutner's concentrated medium is toxic to one of our autotrophic strains of *E. gracilis*, and his dilute medium is not favorable to growth of the same strain.

The case of *Chilomonas paramecium* may possibly be analogous to that of *E. gracilis*. Mast and Pace (1933) have reported that *C. paramecium* is capable of heteroautotrophic

nutrition, while Loefer (1934), Lwoff and Dusi (1934), Pringsheim (1935), and Hall and Loefer (1936) have been unable to duplicate these results. Conceivably, the autotrophic strains of Mast and Pace might have been established as a result of selective adaptation, whereas other workers have been less fortunate in their investigations.

SUMMARY

The establishment of two autotrophic strains of *E. gracilis* is described. Establishment of the autotrophic strains involves, in early transfers, the death of most of the population, while a small proportion of facultative autotrophs becomes adapted to the inorganic media and multiplies to produce healthy autotrophic strains. One strain is now in the fifteenth transfer, and the other is in the thirteenth. One of the strains has been transplanted successfully into three different media, indicating that, once an autotrophic strain is established, it is capable of growth in any one of several satisfactory inorganic media. Attempts to grow an autotrophic strain in Hutner's (1936) media were unsuccessful.

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RELATION BETWEEN THE RESPONSES OF AMOEBA PROTEUS TO ALTERNATING ELECTRIC CURRENT AND SUDDEN ILLUMINATION¹

(One figure)

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MANY organisms react in a definite manner to sudden changes in intensity of a stimulating agent—plants usually by a change in rate of growth, animals by a motor response of some kind. Reactions of this sort are usually referred to as “shock reactions.” Among animals, the amoeba is known to be especially favorable for a study of shock reactions, as it reacts to a number of stimulating agents with a characteristic response, which consists of a cessation of protoplasmic flow. Direct electric current, alternating current, light, mechanical shock—all bring about a definite reaction in this animal.

It has long been known that the amoeba orients in a direct current, moving toward the cathode. Hahnert (1932) found that, if an amoeba is moving toward the anode as the current is turned on, it finally reverses its direction of movement, first stopping for a time, and that the time required to bring about this stoppage depends on the strength of the current, being shorter in a strong current than in a weak one. Mast (1931a) reports that the amoeba usually stops when subjected to an alternating current; and Thomas (1935, 1937), who studied the reaction in some detail, found that, as in the case of a direct current, the reaction-time is longer in a weak current than in a strong one. He found that the reaction-time consists of a stimulation period, during which exposure to alternating current is necessary if an amoeba is to respond and which takes up the first part of the reaction-time, and a latent period, during which exposure is not necessary for a response.

Folger (1925) had already determined the same phases in the response to light by the amoeba that Thomas later found to exist with alternating current. In both cases the amoeba ceased moving upon being stimulated, and the reaction-time became shorter as the intensity of the stimulus increased. In both cases the stimulation period became shorter as the intensity of the stimulus increased; the latent period showed an increase in the length and then a decrease as the intensity of the stimulus increased; the period of quiescence, that is, the time during which the amoeba remain inactive, was found to vary directly as the intensity increased; each reaction is followed by a refractory period, during which there is a decrease in the sensitivity of the animal to stimulation.

Folger (1926) discovered that the responses to mechanical shock are also very much like those to light. Working with light and mechanical shocks, Folger (1927) found that a very close relationship exists between the two. This relationship is demonstrated in his significant finding that an amoeba which has just responded to sudden increase in

¹ Contribution from the Biology Laboratory of Fisk University. The writer wishes to express his gratitude to Professor H. T. Folger, under whose direction the work was done, and to Professor Elmer S. Imes, of the physics department of Fisk University, for invaluable assistance with the electrical equipment.

luminous intensity is rendered less sensitive not only to illumination but also to mechanical shock. The reverse of this was also found to be true.

From the foregoing statement it is to be seen that, when used singly, sudden illumination, alternating current, and mechanical shock produce similar effects on the amoeba. As pointed out, a close interrelationship exists between the response to light and the response to mechanical shock. This paper is concerned with experiments designed to find out if there is a similar relationship between the reactions to light and to alternating current.

MATERIALS AND METHODS

All the observations described in the following pages were made upon specimens of *Amoeba proteus* that were cultivated in glass bowls containing about 100 cc. of a culture solution made by adding raw hay and wheat to distilled water. The hay and wheat soon became covered with a mold, on and among the strands of which aggregated numerous specimens of bacteria and small ciliates, upon which the amoebae fed.

In preparing the amoebae for observation, from ten to twenty of the most active specimens were transferred, by means of a capillary pipette, from the culture solution to a finger bowl of distilled water. They were left in this bowl 15-30 minutes. By this time nearly all of them were radiate in form. The radiate ones were removed and transferred directly to the test solution, which was a modified Ringer's solution devised and described by Chalkley (1929). The experiments were carried on in a darkened room, where the temperature varied little during any experiment.

The electricity used was drawn from a 110-volt alternating-current lighting circuit wired in parallel with a rheostat (*G*) of 922 ohms resistance (see Fig. 1). A glass trough (*H*) was made by cementing thin strips of glass on the surface of a glass slide $2\frac{1}{2}$ inches long and $1\frac{1}{2}$ inches wide. This trough was placed in a wood frame (*K*) of similar inside dimensions on the stage of a compound microscope (*N*), at each end of which was a metal binding post (*J*), to which a platinum electrode (*I*), $1 \times \frac{1}{2}$ inch in size, was attached. These electrodes rested evenly on the bottom of the trough in which were the amoebae to be observed.

By connecting the slide contact (*F*) of the rheostat to one of the electrodes over a key (*L*) and by connecting one of the other two binding-posts of the rheostat directly to the second electrode, as potential divider arrangement was obtained whereby the voltage on the electrodes could be varied at will. The resistance of the test solution was measured by a Wheatstone-bridge method of measuring electrical resistance and was found to be 20,560 ohms. From the equation $I = (E/R)$, where *I* is the current, *E* the voltage, and *R* the resistance, it is seen that the current will vary directly as the voltage *E* if the resistance *R* is kept constant. In the setup for the experiments to be described in this paper the resistance was constant, so that the current did vary directly as the voltage. Owing to the type of setup, it was found that variations in the voltage could be measured more accurately than variations in the current.² By dividing the rheostat into twenty-two equal parts and setting the slide contact on any one of these marks, the voltage could be increased in steps of 5 volts.

The apparatus for the experiments involving light (see Fig. 1) was very similar to that used by Folger (1925). Light was obtained from a 1,000-watt 123-volt Mazda

² When the entire rheostat was included in the setup, the current was approximately 0.005 amperes; and when it was excluded, the current was 0.05 amperes.

stereopticon lamp (*B*), mounted in an asbestos-lined box (*A*). A beam of light, admitted through a circular aperture (*D*), 50 mm. in diameter, was reflected to the stage of the microscope by means of the substage mirror set at an angle of 45° . The light rays were passed through distilled water (*C*) for absorption of heat. The lamp was always turned on only a few moments before the shutter (*E*) was taken from in front of the circular aperture. This gave the lamp filament sufficient time to reach its maximum brightness before it was flashed on the amoebae in the observation trough.

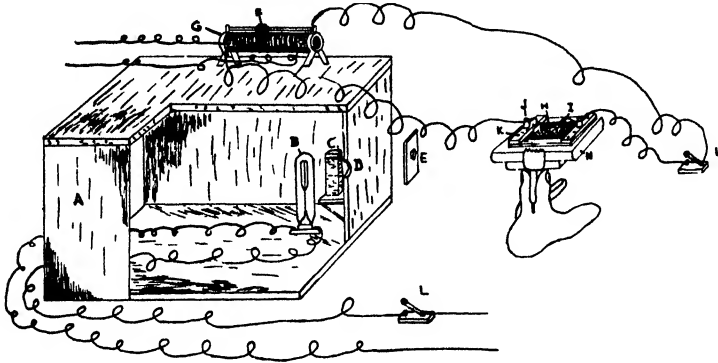


FIG. 1.—Diagram of apparatus used in experiments on amoeba. *A*, asbestos-lined box; *B*, stereopticon lamp; *C*, distilled water; *D*, circular aperture; *E*, shutter; *F*, slide contact of the rheostat; *G*, rheostat; *H*, glass trough; *I*, platinum electrode; *J*, metal binding-post; *K*, wood frame; *L*, switch key; *N*, stage of microscope.

Before an animal was experimented upon, either with light or electricity or the two combined, it was allowed to remain in the dark from $\frac{1}{2}$ to 2 hours, so as to become adapted to the dark and acclimatized to the temperature of the room. A 7.5-watt red electric bulb in a lamp stand furnished sufficient light so that the amoebae might be examined at any time. This red light was equivalent to darkness, having no observable effect on the amoebae. The observations were made with various degrees of magnification, depending on the type of experiment being conducted. When the light was only 25 cm. from the trough, a piece of red glass was put over the ocular of the microscope to protect the eyes.

EXPERIMENTAL RESULTS

I. REFRACTORY PERIODS

a) Variations in reaction-time after various refractory periods for light.—Folger (1925) has shown that after an amoeba has been subjected to sudden increase in light intensity and has responded to this stimulus, a certain amount of time is required for recovery from the effects of the stimulus before the amoeba will again respond in the same manner to a second illumination. If the time for recovery is very short, there will be no response; if the time is somewhat longer, the amoeba may respond, but with an increased reaction-time. Ultimately, usually within $1\frac{1}{2}$ minutes, the amoeba will have completely recovered and will again respond in the usual manner. The time during which the amoeba shows a decrease in sensitiveness to light stimulus is apparently analogous to the refractory period found in the response of nerve. During this time the animal is recovering from

the effects of the stimulation. The general term "recovery period" has usually been used in this paper to indicate the time between stimulations. In the following preliminary experiment the work of Folger has been repeated and paralleled.

An actively moving amoeba, preferably monopodal, was brought to the center of the field of the microscope, which was so placed that the light was 24 cm. from the experimental trough. The animal was exposed to illumination; the reaction-time was obtained; and then, after a recovery period of 90 seconds, the amoeba was again stimulated and the reaction-time ascertained. Ninety seconds were found to be sufficient for complete recovery in every amoeba used, as shown by the fact that a recovery period of longer than 90 seconds produced no shorter reaction-time. After finding the reaction-time following complete recovery, one could begin the experiment proper, which was to show the effect of various shorter recovery periods on the reaction-time. A complete experiment consisted of (1) stimulation after 90 seconds for recovery, followed by (2) stimulation after various periods ranging from 60 seconds to 7.5 seconds and (3) ascertaining the reaction-time after each stimulation. Three or four readings were taken for the reaction-time after each recovery period and averaged. The results of three experiments of this type are presented in Table 1.

Examination of Table 1 shows that the results obtained in the various individual tests for the three specimens used were fairly consistent. In each case, decided increases in reaction-times were seen as the time allowed for recovery was shortened. Considering the results obtained with amoeba No. 2, one sees that its reaction-time after a 90-second recovery period was 1.86 seconds; after a 60-second recovery period, 3.7 seconds; after a 45-second recovery period, 4.33 seconds; and after a 15-second recovery period, 6.7 seconds. Further examination of the table shows that none of the three animals reacted after recovery periods shorter than 15 seconds. In the cases of a few amoebae experimented with, 60 and 45 seconds were found to be sufficient for complete recovery, as shown by the fact that the average reaction-time following 60 and 45 seconds was approximately the same as that following 90 seconds. The results for every amoeba used showed the same general result, though, as may be seen from the table, there was considerable variation in the average reaction-times of the several animals used.

From these results the following conclusion can clearly be drawn. A certain amount of time, specifically 90 seconds or so, is necessary for an amoeba to completely recover from the effect of stimulation by light. A shorter time for recovery results in an increase in reaction-time. If the time for recovery is made less than 15 seconds, the animal refuses to respond at all.

b) Variations in reaction-time for alternating current after various refractory periods.—In his work on the effect of alternating current on amoeba, Thomas (1935) did not ascertain in detail the effect of insufficient recovery on the reaction-time. As mentioned before, in various other respects, his results were similar to those obtained by Folger with light and corresponding types of experiments. One might expect, then, that the relation between reaction-time and recovery period would be similar for the two stimulating agents. Table 2 presents the results obtained from an experiment designed to ascertain whether or not this is true. The procedure for this experiment was identical with that for the one just described, except that stimulation by light was replaced with stimulation by alternating current. The voltage on the electrodes was kept at 75 volts, this voltage having been found to be the most favorable for the purpose of the experiment. A re-

covery period of 90 seconds was sufficient for all the amoebae to completely recover from previous stimulation by alternating current.

TABLE 1
RELATION OF RECOVERY PERIOD AND REACTION-TIME FOR LIGHT

Amoeba Number	Recovery Period Allowed (Seconds)	Average Reaction-Time (Seconds)
1.....	90	2.6
	60	3.3
	45	3.9
	30	4.57
	15	5.7
	7.5	No reaction
2.....	90	1.86
	60	3.7
	45	4.33
	30	4.83
	15	6.7
	7.5	No reaction
3.....	90	2.0
	60	3.6
	45	4.1
	30	4.2
	15	5.2
	7.5	No reaction

TABLE 2
RELATION OF REACTION-TIME TO RECOVERY PERIOD
FOR ALTERNATING CURRENT

Amoeba Number	Recovery Period Allowed (Seconds)	Average Reaction-time (Seconds)
1.....	90	1.1
	60	1.1
	45	1.2
	30	3.0
	15	11.0
	7.5	No reaction
2.....	90	1.15
	60	1.31
	45	1.8
	30	3.0
	15	8.35
	7.5	No reaction

From Table 2 it is seen that amoeba No. 2, for instance, gave a reaction-time of 1.15 seconds after 90 seconds for recovery. After 60 seconds for recovery, the reaction-time was 1.31 seconds; after 45 seconds, 1.8 seconds; after 30 seconds, 3.0 seconds; after 15

seconds, 8.35 seconds. There was no reaction after recovery periods shorter than 7.5 seconds.

The similarity between the results of this experiment and the preceding one, where light was used as the stimulating agent, is evident. In both cases an insufficient time for recovery brings about an increase in reaction-time.

II. THE RELATIONSHIP OF SUDDEN ILLUMINATION AND ALTERNATING CURRENT

a) *Changes in reaction-time for stimulation by light after various refractory periods following stimulation by alternating current.*—The two preceding experiments show most clearly that reaction-time and recovery period have the same relation for *A. proteus* whether the stimulating agent is sudden illumination or alternating current. As mentioned above, Folger (1927) found that a mechanical shock preceding sudden illumination influences the response to the latter. Insufficient recovery from mechanical shock, followed by sudden illumination, resulted in a longer reaction-time or no reaction at all. The reverse of this was also found to be true. Since responses in amoebae to sudden illumination and alternating current are so similar, two questions arise: Does sudden illumination affect the response to alternating current? If so, is the reverse true? The following two experiments are designed to answer these questions.

As may readily be seen from the description of the apparatus used for stimulation by light and alternating current, the two separate setups could easily be used together. In the first experiment an amoeba was stimulated by alternating current over a voltage of 75 and was then allowed to recover from its effects for various lengths of time. At the end of these periods, which ranged from 90 to 7.5 seconds, it was exposed to light from the lamp, which was placed at a distance of 50 cm. from the experimental animal. Stimulation by alternating current was always preceded by a period of not less than 90 seconds, which, as we have already seen, permits complete recovery. Table 3 presents the results of such an experiment.

Examination of Table 3 shows that alternating current does have an effect upon the response in amoeba to sudden illumination but that this effect is diametrically opposed to that which mechanical shock has upon response to light, as maintained by Folger. From the results recorded for amoeba No. 1, one sees an average response to light of 1.83 seconds after 90 seconds for recovery. This reaction-time was one of the quickest noted in any amoeba for light after a complete recovery. Yet, as may be seen from Table 3, when this particular amoeba was stimulated by light immediately after a previous stimulation by alternating current, the reaction-time became even shorter. Stimulation by light following a recovery period of 60 seconds gave a reaction-time of 1.70 seconds; following a recovery period of 45 seconds, a reaction-time of 1.56 seconds; following a recovery period of 30 seconds, a reaction-time of 1.36 seconds; following a recovery period of 7.5 seconds, a reaction-time of 1.10 seconds. Further observation of the table reveals that amoebae Nos. 2 and 3 show exactly the same effect. The decrease in reaction-time with decrease in time allowed for recovery from the effect of alternating current is very marked in each experiment. Clearly, alternating current makes an amoeba more sensitive to light.

b) *Changes in reaction-time for alternating current after various refractory periods from sudden illumination.*—The following experiment was designed to ascertain what effect light has on the response to alternating current. The method of procedure was the reverse of that described for the preceding experiment. An amoeba was first exposed to

light, placed at a distance of 25 cm., and then allowed to recover from its effects for various lengths of time, after which it was subjected to alternating current, the voltage on the electrodes being 60.

The results tabulated in Table 4 are typical of those obtained with numerous amoebae. The two amoebae considered were chosen because their reaction-times after complete recovery were about the same. As may be seen from Table 4, amoeba No. 1 gave a reaction-time of 2.5 seconds after 90 seconds for recovery. After 60 seconds for recovery,

TABLE 3
EFFECT OF ALTERNATING CURRENT UPON RESPONSE
TO SUDDEN ILLUMINATION

Amoeba Number	Time after Stimulation by Alternating Current before Stimulation by Light (Seconds)	Average Reaction-Time for Light (Seconds)
1	90	1.83
	60	1.70
	45	1.56
	30	1.36
	15	1.25
	7.5	1.10
2	90	2.50
	60	2.26
	45	1.80
	30	1.50
	15	1.32
	7.5	1.03
3	90	2.76
	60	2.50
	45	2.20
	30	1.80
	15	1.50
	7.5	1.20

the reaction-time had decreased to 1.2 seconds; after 45 seconds for recovery, to 1.0 second; after 30 seconds for recovery, to 0.9 second; and after 15 seconds for recovery, to 0.7 second. Amoeba No. 2 showed the same sort of result.

Thus, it is evident that, just as exposure to alternating current sensitizes the amoeba to light, so light sensitizes it to alternating current.

c) *Summation effects of stimuli below the threshold intensity.*—Folger (1927) found that, if an amoeba is stimulated by a light which of itself is insufficient to bring about a reaction, and then immediately after by a mechanical shock, also too slight to bring about a reaction alone, there may be a summation effect; the two subminimal stimuli, working together, bring about a reaction. This has been taken to mean that the effects of light and mechanical shock are the same. As demonstrated in the preceding pages, the relationship between the effects of light and alternating current are not the same as between light and mechanical shock. If an amoeba is subjected to mechanical shock, it must undergo a recovery before it will respond to illumination. On the other hand, if

it is subjected to alternating current, not only does it not require a period of recovery before it will respond to light, but it responds even more readily than if it had not been stimulated by electric current. It should be of interest to note the effect of the two stimulating agents when used concurrently, each being made so small as to have no visible effect by itself.

TABLE 4
EFFECT OF SUDDEN ILLUMINATION UPON THE RESPONSE
TO ALTERNATING CURRENT

Amoeba Number	Time between Stimulation by Light and Alternating Current	Average Reaction-Time for Alternating Current
1.....	90	2.5
	60	1.2
	45	1.0
	30	0.9
	15	0.7
2.....	90	2.4
	60	1.8
	45	1.4
	30	1.2
	15	1.0

TABLE 5
EFFECT OF ALTERNATING CURRENT UPON THE RESPONSE TO LIGHT
BELOW THE INTENSITY THRESHOLD

Amoeba Number		Number of Trials	No Reactions
1.....	Sudden illumination alone	3	3
	Sudden illumination following immediately after shock by alternating current	8	3
2.....	Sudden illumination alone	6	6
	Sudden illumination following immediately after shock by alternating current	4	0
3.....	Sudden illumination alone	6	6
	Sudden illumination following immediately after shock by alternating current	6	1
Totals..	Sudden illumination alone	15	15
	Sudden illumination following after shock by alternating current	20	4

Table 5 shows the results of such an experiment. In this experiment subminimal stimuli, both of light and alternating current, were employed, one being applied immediately after the other. As indicated, no reactions at all were obtained when either of the two types of stimuli were used alone. But when the two were used together, one immediately after the other, reactions usually occurred. When alternating current was

used first, followed immediately by sudden illumination, sixteen reactions were obtained out of twenty trials. When sudden illumination was used first, followed immediately by alternating current, eleven reactions were obtained out of sixteen trials.

These results are not at all unexpected. However, their similarity with those of Folger, just referred to, is, perhaps, somewhat misleading. Apparently, light and mechanical shock have the same effect on amoebae. The evidence seems to show that, when an amoeba is subjected to a short period of illumination and then to a slight mechanical shock, its condition is just as it would have been if another short period of illumination had been substituted for the mechanical shock. From what has been given in the preceding experiments it is clear that light, followed by alternating current, does not have the same effect as a longer, single exposure to light. The amoeba is not affected by the alternating current in just the same way as by the light but, having been sensitized by the latter, responds to a current that is ordinarily below the threshold intensity.

DISCUSSION

Involving, as they do, a cessation of movement, any explanation of the reactions and relationships that have been under consideration must be concerned with the mechanics of amoeboid movement. According to Mast (1926), the essential structure of amoeba, so far as movement is concerned, consists of a liquid inner portion, designated the "plasmasol," which is surrounded by a more solid layer, the "plasmagel." A very thin layer, the "plasmalemma," surrounds the entire animal but, for our purposes, may be ignored. The plasmagel is elastic; and being subjected to a pressure, probably osmotic, by the plasmasol, is stretched. If it were of the same strength throughout, the amoeba would assume a spherical form. Ordinarily, of course, this is not true. Actually the plasmagel is usually unequal in strength at various points and is continuously changing in the living animal, resulting in the variety of shapes so characteristic of the amoeba. In a moving amoeba the plasmagel is very thin and weak at the anterior end, so that it is continuously bulging at this point.³

It is evident that a certain equilibrium must exist between internal pressure and the relative strength of the plasmagel at various points on the amoeba. Any changes, either in internal pressure or in the plasmagel, must alter this equilibrium. The effect of both light and alternating current (Mast, 1931a and 1931b), and presumably mechanical shock, is to coagulate the thin sheet of plasmagel at the end of the advancing pseudopodium and thus prevent it from being pushed forward. As a result, the animal ceases to move. But even after movement is resumed, the equilibrium which is at first established is different from that which existed before the animal was stimulated. Hence the refractory period. Gradually the old equilibrium is re-established; and when this occurs, the refractory period is at an end.

But what of the relationships between the various stimulating agents that have been noted? Mast gives evidence (1932, p. 5) for believing that light strengthens the plasmagel not only at the tip of the advancing pseudopodium but at all other points as well. Thus, the general effect of light is to increase the elastic strength of the plasmagel. Recovery from its effects, then, we may assume, includes a lowering in the strength of the plasmagel. It takes some time, even after the light has been turned off, for the plasmagel

³ Of course, other factors are concerned in amoeboid movement, but they need not concern us here. For further information the reader is referred to Mast's (1926) account.

to return to its former condition. Until it does, the amoeba is less sensitive to stimulation by light. Assuming that mechanical shock has the same effect on the plasmagel that light has, it is evident that mechanical shock should make an amoeba less sensitive not only to another mechanical shock but to light as well. Likewise, light should make an amoeba less sensitive to mechanical shock. Electricity, however, has an effect in many respects different from that of light. If an amoeba is placed in a direct current but with its anterior end facing the anode, it reverses its direction of flow, the anterior end now facing the cathode. Thus, the current has caused coagulation of the plasmagel at the one end of the amoeba, but at the same time it has caused solation of the plasmagel at the other end. Mast (1931a) has noted a similar effect with alternating current. He says (1931a), p. 326):

The evidence presented in reference to the effect on *Amoeba proteus* of both the direct and the alternating current indicates that the assertion . . . that electricity gels cytoplasm, is misleading, for it shows that if an electric current causes gelation in a cell it probably always causes simultaneous solation, each being confined to a portion of the cell

Thus we may conclude that, while alternating current causes increase in strength of the plasmagel at some points on the amoeba, it causes a decrease at other points. The general effect is probably a lowering of the strength of the plasmagel. Recovery from the effects of alternating current, then, includes a general increase in the strength of the plasmagel. During the refractory period following alternating current the amoeba is less sensitive to alternating current; but, as the effect of the current is—in some respects, at least—opposed to that of light, it is more sensitive to light. Light, of course, should and does have the same effect on response to alternating current.

SUMMARY

1. Amoebae react both to light and to alternating current by a cessation of movement, which takes place after an appreciable reaction-time.
2. In both cases the reaction-time is dependent on the magnitude of the stimulus, becoming longer as the magnitude decreases.
3. In both cases the reaction is followed by a distinct refractory period, during which the amoeba is less sensitive to stimulation, as indicated by failure to respond a second time, or, if it does respond, by an increase in reaction-time. However, within a short time, usually within 90 seconds, the amoeba has completely recovered, and again responds as at first.
4. Folger has reported that, during the refractory period which follows response to light, the amoeba is less sensitive not only to light but also to mechanical shock. The reverse of this is also true. However, during the refractory period following response to light, an amoeba is not less sensitive, but more sensitive, to alternating current, and responds to the current with a reaction-time that is shorter than it would have been if the stimulation by electricity had not been preceded by exposure to light. Likewise, exposure to alternating current renders the amoeba more sensitive to light.
5. The effect that one of these stimulating agents has on the other is also shown by the use of subminimal stimuli. If the amoeba is subjected to one of these stimulating agents that is too slight in itself to bring about a reaction, it may respond if it has been sensitized by the other stimulating agent, even though that also is too slight to bring about a reaction.

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ERRATUM

In Volume XI, page 362, Table 1, last columns, change 10.50 ± 0.44 to 10.50 ± 0.27 and 3.98 to 2.42.

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No. 2

INITIATION OF MATURATION IN THE FROG EGG

(One plate)

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WHEN an immature egg is induced to begin maturation, it does so as the result of a stimulus. The stimulus to maturation has often been studied in the case of invertebrate eggs; and it is well known that various types of physical and chemical agents can cause the initiation of maturation in the eggs of annelids, mollusks, etc. Recently the initiation of maturation in the egg of the annelid *Nereis* has been studied from the standpoint of the calcium-release theory of stimulation, and evidence has been presented which indicates that the breakdown of the nuclear membrane in this egg is conditioned by calcium release from the egg cortex (Heilbrunn and Wilbur, 1937).

As yet, the initiation of maturation in the frog egg is not well understood; and, as a matter of fact, the problem has scarcely been attacked on an experimental basis. It was thought that frog eggs might be studied in somewhat the same manner that invertebrate eggs have been studied. Eventually perhaps, information might be obtained which would link up the phenomena in frog and other vertebrate eggs with the facts observed and the theories proposed for invertebrate eggs. The results might also have some significance for the general protoplasmic theory of stimulation.

Immature frog eggs are easy to obtain. At most seasons of the year frog ovaries contain many large eggs, and many of these are ready to mature and to be released from the ovary. Indeed, in the case of *Rana pipiens*, Wolf (1929) and Rugh (1934) showed that implantation or injection of pituitary would cause these ovarian eggs to break through the ovarian wall and to pass into and through the oviduct. They lodge in the uterus, and they may then be squeezed out of the frog and fertilized. Presumably, therefore, the eggs which have been caused to ovulate and to pass through the oviduct as a result of pituitary treatment have begun their maturation, for normally in the frog, fertilization occurs only after maturation has begun.

In our experiments we attempted to discover the nature of the stimulus which causes the immature frog egg to begin its maturation. Our results also throw some light on the factors concerned in the release of the egg from the ovary. Rugh (1935) found that, when an excised frog ovary is immersed in Ringer's fluid, or in Ringer's fluid to which anterior

pituitary hormone has been added, the eggs remain in the ovary and do not escape. However, when he added pepsin and dilute hydrochloric acid to Ringer's fluid, such a digestive solution caused the eggs to escape in 5 minutes. Rugh therefore suggested the possibility that the anterior pituitary hormone might supply "an activator for a digestive ferment, originating in the egg and acting on the follicle wall." Since Rugh's experiments Zwarenstein (1937) has stated that addition of the mammalian hormone progesterone causes extrusion of ova from the excised ovaries of the African clawed toad, *Xenopus laevis*. The progesterone was added to Ringer's fluid in which the ovaries were bathed, and the ova were extruded in 8-10 hours.

Our results do not confirm Rugh's statement that pituitary hormone is incapable of causing the eggs to be extruded through the walls of the ovary. We prepared an extract by mashing a pituitary gland in 1 cc. of Ringer's fluid. To this was then added 29 cc. of Ringer's fluid. When ovaries were immersed in such a suspension of pituitary gland, it was noted that, after the lapse of several hours, the eggs could be observed in large numbers escaping through the wall of the ovary. For example, in experiments performed on the excised ovaries of two females (*R. pipiens*) the number of eggs extruded during 24 hours' immersion was found to be 203 and 280, respectively. Similar treatment of the excised ovaries of another female (*Rana clamitans*) resulted in the release of 101 eggs during the first 24 hours and 193 eggs during the following 24-hour period. On the other hand, when the ovaries of either species of frog were immersed in Ringer's fluid without any admixture of pituitary, no extrusion of eggs occurred. These experiments were performed in the spring of the year.

The ovarian frog egg, like immature eggs generally, possesses a large nucleus, or germinal vesicle. Our experiments are primarily concerned with the attempt to determine the stimulus to breakdown of this vesicle. Unfortunately, the eggs, as they reach their definitive size, become thoroughly opaque, so that it is not a simple matter to determine whether or not the germinal vesicle is broken down; nor can one easily follow the course of the maturation divisions. It is true that with a little practice one can dissect out the germinal vesicle from frog eggs, and in this way one might be able to decide whether the vesicle is intact. Such a method is rather uncertain, for every now and again the vesicle is injured in the dissection process, and the injured vesicles are apt to become unrecognizable. The only sure way to discover what has happened to the germinal vesicle in a given experiment is to section the eggs with ordinary histological techniques. In such sectioned eggs it is possible, without much difficulty, to make counts of intact and broken-down germinal vesicles; and one can thus determine accurately the percentage of eggs which has responded to treatment in any given experiment. The frog egg is not easy to section; and, indeed, it is well known to be unusually refractory for ordinary sectioning methods. In our studies fixation was carried out in Zenker's or Smith's fluid (see Smith, 1915). Fixation in Smith's fluid was found to be improved by reducing the acetic acid to half the amount prescribed. The sections were stained in borax carmine.

Eggs of *R. pipiens* were dissected from the ovaries in Ringer's solution. In our first experiments we tried the effect of ultra-violet radiation from a mercury arc lamp. Such radiation is very effective in inducing maturation in the egg of *Nereis*. However, no clear-cut results were obtained with the frog egg.

Obviously, therefore, the egg of the frog does not behave in the same manner as do invertebrate eggs. We were led to try the effect of pituitary extract in order to determine

whether or not this might have an effect in inducing maturation. The eggs were exposed to pituitary extracts, prepared as noted above. They were left in contact with the extracts for periods of either 24 or 48 hours. Control eggs were left in Ringer's fluid for similar periods of time. At the expiration of the experiment the eggs were sectioned, and counts were made of the percentages of intact and broken-down germinal vesicles. These counts are shown in Table 1. It is clear that no germinal-vesicle breakdown occurs in the control eggs in Ringer's fluid. On the other hand, in the eggs treated with pituitary

TABLE 1

No OF FROG	RINGER'S SOLUTION		RINGER'S SOLUTION — PITUITARY EXTRACT			
	No of Eggs with Intact Germinal Vesicles	No of Eggs Showing Ger minal Vesicle Breakdown	No of Eggs with Intact Germinal Vesicles after 24 Hours' Treatment	No of Eggs Showing Ger minal Vesicle Breakdown after 24 Hours' Treatment	No of Eggs with Intact Germinal Vesicles after 48 Hours' Treatment	No of Eggs Showing Ves icle Breakdown after 48 Hours' Treatment
1	20	0			1	17
2			6	22		
3	18	0	0	39		
4	27	0	25	0		
5	22				21	5
6	27	0			28	0
7	25	1*			10	16
8	26	0			3	21
9	25	0			10	9
10	25	0			16	11
11†	23	0			17	9
12†	28	0	10	11		
	31				16	17
	28	0	25	0		
	24				23	2
Total	140	1*	66	72	151	107
Percentage	100		47.8	52.2	58.6	41.4

* Egg abnormal in appearance

† Holtfreter's solution was used in place of Ringer's solution

there is a rather high percentage of breakdown. This percentage would have been higher if it were not for the fact that certain frogs contain eggs which seem to be particularly refractory to treatment. Thus, in the case of frog No. 5, not a single one of the eggs showed germinal-vesicle breakdown. The broken-down germinal vesicles often showed a characteristic appearance in the sectioned material. This is illustrated in Plate I, Figure 1. As another control, eggs from Frog No. 7 were immersed in a suspension of macerated gastrocnemius muscle and Ringer's solution. Of 32 eggs examined, all showed intact germinal vesicles after 48 hours' immersion in this suspension.

It might be thought that the breakdown of the germinal vesicle induced by pituitary extract is a pathological process which bears no relation to normal maturation. This is certainly not true, for some of the eggs treated with pituitary extract undergo maturation

divisions and show polar bodies. This is illustrated in Plate I, Figures 2 and 3, which show a polar body and two maturation spindles.

In conclusion, it may be stated with certainty that pituitary extracts are capable of initiating the maturation process in the frog egg. These extracts produce the same sort of effect that irradiation and various chemical stimulants produce on the egg of *Nereis*. Although the immature frog egg is not affected by the same stimulating agents as those which induce maturation in invertebrate eggs, it is a matter of interest that the one factor we have found to be effective for frog eggs may also be regarded as a rather general stimulating agent. At any rate, the anterior pituitary hormone causes a contraction of the smooth muscle of vertebrates. It is clear, therefore, that the initiation of maturation in the frog egg can be the result of a stimulus which can affect other types of irritable systems. As to whether in the intact frog maturation is induced as a result of the presence of pituitary extract in the blood, we offer no opinion. It is quite possible that another factor or factors may be involved.¹

SUMMARY

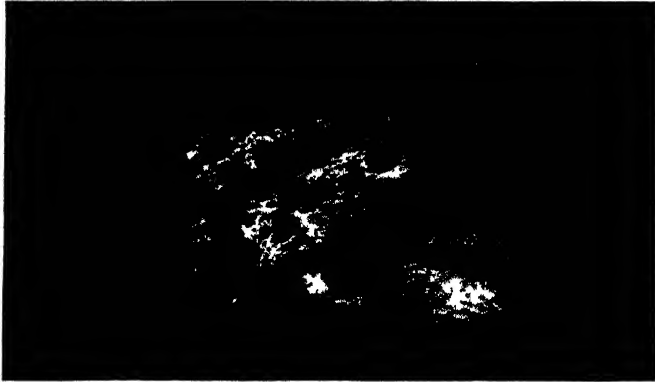
Extracts of the pituitary gland of the frog cause the initiation of maturation in the frog egg. These extracts also induce the release of eggs from the ovary.

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¹ We are grateful to Dr. C. L. Parmenter for instruction in the frog-pituitary technique and for advice and help in the interpretation of the sectioned material.

PLATE I



1



2



3

PLATE I

FIG. 1.—Section of an egg treated with pituitary extracts showing the broken-down germinal vesicle. $\times 60$.

FIG. 2.—Section of an egg treated with pituitary extracts showing the polar body and a second maturation-division spindle (*s*). $\times 870$.

FIG. 3.—Section of an egg treated with pituitary extracts showing the spindle. $\times 500$.

These eggs were fixed with Zenker's fixative and stained with borax carmine.

THE RELATION OF THE MAGNESIUM ION TO ULTRA-VIOLET STIMULATION IN THE NEREIS EGG¹

(Two figures)

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THE *Nereis* egg, after being shed into sea water, rests inert with its germinal vesicle intact until it is fertilized, after which the germinal vesicle breaks down. This breakdown of the germinal vesicle can be produced artificially by treating the egg with a variety of stimulating agents, such as ultra-violet light (Just, 1933) and isotonic solutions of NaCl and KCl. Accordingly, the *Nereis* egg provides very favorable material for the study of the mechanism of response, for one has a perfectly definite reaction, that of nuclear breakdown, which can be easily followed and which takes place in a relatively simple protoplasmic system. This reaction is apparently dependent upon the calcium ion, for, if the calcium is first removed from the egg cortex by treating the egg with sodium citrate, the stimulating agents which ordinarily produce germinal-vesicle breakdown are ineffective. It has been suggested that NaCl, KCl, and other stimulating agents act by releasing calcium from the cell cortex and that this is one step in a series of reactions leading to the breakdown of the germinal vesicle (Heilbrunn and Wilbur, 1937).

Now, it is an interesting fact that in sea water there are three cations—sodium, potassium, and calcium—which in one way or another favor stimulation; and yet in sea water the germinal vesicle does not break down spontaneously. Perhaps the fourth, and only other common cation, magnesium, actually inhibits spontaneous breakdown in sea water. It is, therefore, of interest to determine, on the one hand, the relation of magnesium to the other cations in the normal environment and, on the other, the relation of magnesium to nuclear breakdown when it is produced by a stimulating agent. Ultra-violet radiation was used as the stimulating agent, and as a quantitative index of response the percentage of nuclear breakdown was determined.

METHOD

The usual procedure employed was to transfer 1 drop of eggs in sea water to 20 cc. of the experimental solution. Approximately 1 cc. of this solution containing eggs was then placed in each of a series of dishes 3.3 cm. in diameter. After 15–20 minutes' immersion in the experimental solution the eggs were exposed to the unfiltered radiation of a mercury arc lamp at a distance of 23–24 cm. The lamp was a Uviarc Laboratory Outfit operating at 120–50 volts and 5.5–4.0 amperes.² The energy output was not of interest in this study and was not determined. An electric fan was used with the lamp

¹ This study was carried out under the direction of Dr. L. V. Heilbrunn. I gratefully acknowledge his kindness in offering suggestions and criticisms throughout the experimental work and the preparation of the manuscript. I am also indebted to Mr. F. J. Thomas for help with certain portions of the work.

² This is the same lamp used in earlier experiments (Heilbrunn and Wilbur, 1937), but the values given in that paper for voltage and amperage are not correct.

to prevent overheating of the eggs. Room temperature varied between 22° and 26.7° C. On irradiation there was an increase in the temperature of the solution surrounding the eggs of approximately 3° with an exposure of 45 seconds. A 30-second exposure gave an elevation of approximately 2.5° , with less than 1.5° for 10-second irradiation.

The percentage of nuclear breakdown was determined after a period of 1 hour, or longer, following irradiation. In order to make accurate counts the eggs were compressed beneath a cover slip. Each point on the graphs represents the average of ten determinations, and each of the ten determinations was made for a different individual. In every case at least one hundred eggs were counted. The characteristics of the lamp given above indicate that the energy output was not uniform throughout the experiments; in fact, slight variations could be observed from minute to minute. This, together with the variation of the material itself, would necessarily result in differences in the values obtained for different lots of eggs with the same time of exposure. However, the results for the several animals of each experiment were strikingly consistent in that they were always in the same direction. And there is no instance in which any one lot of eggs behaved in a different manner from those of the rest of the series.

RESULTS

Figure 1 indicates the inhibiting action of $MgCl_2$ when eggs are irradiated in a solution consisting of 1 part isotonic $MgCl_2$ to 4 parts of sea water. It will be noted that, when eggs are irradiated 10 seconds in a solution containing excess magnesium, there is not nearly so much germinal-vesicle breakdown as on irradiation in sea water. However, with a longer time of irradiation (20 and 30 seconds) the $MgCl_2$ does not inhibit the action of ultra-violet radiation. This is similar to the reaction which other systems show in the presence of anesthetics, in that a response is obtained if the stimulus is sufficiently increased.

If the concentration of magnesium is further increased (1 part $MgCl_2$ to 1 part sea water), the inhibitory effect is likewise increased, and the irradiation does not overcome the effect of the $MgCl_2$, even with an exposure of 30 seconds (Fig. 2). With a still further increase in the magnesium concentration (4 parts $MgCl_2$ to 1 part sea water), there is actually a greater percentage of breakdown than with the lower concentration; and with 30 seconds' irradiation the inhibitory action is almost completely overcome.

It is surprising to find that 45 seconds' irradiation in sea water produces a lower percentage of germinal-vesicle breakdown than 30 or 15 seconds' irradiation (Fig. 2).

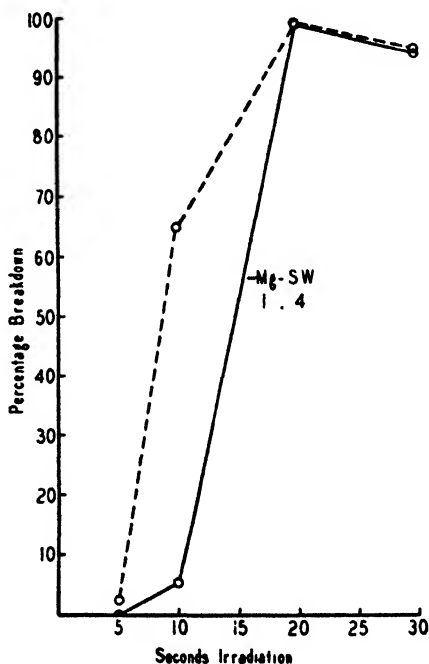


FIG. 1.—The percentage of germinal-vesicle breakdown following different periods of ultra-violet irradiation. The solid line gives results for eggs in a mixture of 1 part of isotonic $MgCl_2$ to 4 parts of sea water. The broken line gives results for eggs in sea water.

Cytolysis, often present with shorter exposures, is extreme with 45 seconds' irradiation. However, eggs in the MgCl_2 -sea-water mixture do now show this decrease in the percentage of germinal-vesicle breakdown. Whether these eggs in the MgCl_2 -sea-water mixture would likewise show a decrease in percentage of breakdown if exposed longer than 45 seconds has not been determined.

When eggs are subjected to excess magnesium, they are not injured. At any rate, on return to sea water the eggs react to ultra-violet stimulation in normal fashion. Thus, the action of MgCl_2 is perfectly reversible. This was shown in the following manner.

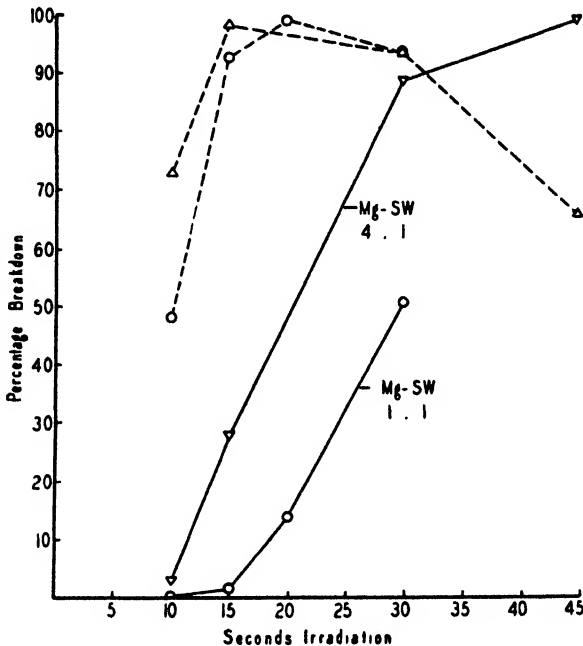


FIG. 2.—The solid line with circles indicates the percentage of germinal-vesicle breakdown following different periods of ultra-violet irradiation in a mixture of 1 part of MgCl_2 to 1 part of sea water. The solid line with triangles indicates results for a mixture of 4 parts of MgCl_2 to 1 part of sea water. The broken lines with circles and triangles indicate the corresponding sea-water controls.

Eggs were immersed in a MgCl_2 -sea-water mixture (1 part MgCl_2 to 1 part sea water) for 19½–21 minutes and then returned to sea water for a similar period. Another sample of eggs from the same female was treated only with sea water. Both samples were irradiated for 10 seconds, and the germinal-vesicle breakdown was compared. Table 1 shows that the percentage of breakdown is as great for the eggs previously treated with the MgCl_2 -sea-water mixture as for the sea-water controls. Similar results were obtained in five experiments with a mixture consisting of 4 parts of MgCl_2 to 1 part of sea water. The average breakdown with 10 seconds' irradiation was 99.2 per cent for the eggs previously treated with the MgCl_2 -sea-water mixture, as compared with an average of 97 per cent for the sea-water controls. These results eliminate the possibility that MgCl_2

may have inhibited the action of ultra-violet radiation by injuring the egg and thus prevented it from reacting.

The changes which take place in the egg as the result of irradiation are not instantaneous. The lifting-off of a membrane, the first obvious change following irradiation, requires a few minutes for completion. Cytolysis and other changes may occur, and after several hours some of the irradiated eggs may disintegrate.³ Since all these

TABLE 1
REVERSIBILITY OF THE INHIBITORY EFFECT OF MAGNESIUM CHLORIDE

No. of Expt.	Percentage of Breakdown in Sea Water Following Magnesium Treatment	Percentage of Breakdown in Sea-Water Control	No. of Expt.	Percentage of Breakdown in Sea Water Following Magnesium Treatment	Percentage of Breakdown in Sea-Water Control
1.....	76	81	6.....	85	85
2.....	100	100	7.....	99	96
3.....	89	96	8.....	100	90
4.....	100	100	9.....	100	100
5.....	55	63	Av.....	89.3	90.1

TABLE 2
INHIBITORY EFFECT OF MAGNESIUM CHLORIDE ADDED TO EGGS SUBSEQUENT TO IRRADIATION

No. of Expt.	Percentage of Breakdown Following the Addition of MgCl ₂ to Sea Water Subsequent to Irradiation	Percentage of Breakdown in Sea-Water Control	No. of Expt.	Percentage of Breakdown Following the Addition of MgCl ₂ to Sea Water Subsequent to Irradiation	Percentage of Breakdown in Sea-Water Control
1.....	0	0	6.....	0	0
2.....	0	78	7.....	0	17
3.....	0	24	8.....	0	38
4.....	0	0	9.....	0	36
5.....	0	12	10.....	0	35

changes require some time for completion, the possibility existed that MgCl₂ might inhibit germinal-vesicle breakdown if added after irradiation. To test this, eggs were irradiated for 10 seconds in sea water, and MgCl₂ was added 3-5 seconds later. (Four cubic centimeters of a mixture consisting of 2.5 parts of MgCl₂ to 1.5 parts of sea water were added to 1 cc. of sea water containing the eggs.) Table 2 shows the results. There is not a single case of breakdown in the eggs in which MgCl₂ was added. It is clear that the addition of MgCl₂ subsequent to irradiation prevents nuclear breakdown.

³ If eggs are irradiated in sea water or a MgCl₂-sea-water mixture (1 part MgCl₂ to 1 part sea water) and counted after 1 hour and then again after several hours, the percentage of germinal-vesicle breakdown may be found to increase on standing.

In order to determine whether the customary calcium-magnesium antagonism would be exhibited by this system, eggs were irradiated for 10 seconds in a MgCl_2 -sea-water mixture containing a quantity of MgCl_2 sufficient to inhibit germinal-vesicle breakdown and compared with eggs irradiated in a mixture containing the same amount of MgCl_2 but with CaCl_2 added to give the calcium-magnesium ratio found in sea water (Table 3). With the MgCl_2 -sea-water mixture there is the usual absence of breakdown. But when CaCl_2 is added, the percentage of breakdown is increased, although not as much as in the sea water alone. There is thus evidence of a calcium-magnesium antagonism.

TABLE 3
THE COUNTERACTING EFFECT OF CALCIUM CHLORIDE ON THE
INHIBITORY ACTION OF MAGNESIUM CHLORIDE

No. of Expt.	Percentage of Breakdown in Sea-Water- MgCl_2 Solution	Percentage of Breakdown in Sea-Water- MgCl_2 - CaCl_2 Solution	Percentage of Breakdown in Sea Water	No. of Expt.	Percentage of Breakdown in Sea-Water- MgCl_2 Solution	Percentage of Breakdown in Sea-Water- MgCl_2 - CaCl_2 Solution	Percentage of Breakdown in Sea Water
1.....	0	21	99	9.....	2	98	100
2.....	0	49	98	10.....	0	0	39
3.....	1	40	92	11.....	0	11	99
4.....	0	15	100	12.....	0	0	12
5.....	0	19	96	13.....	0	47	100
6.....	0	0	63	14.....	0	2	89
7.....	0	0	73	15.....	1	90	100
8.....	0	0	43	Av...	0.3	26.1	80.2

Since magnesium in a concentration higher than that found in sea water inhibits germinal-vesicle breakdown resulting from ultra-violet irradiation, one might logically suppose that the magnesium present in normal sea water is likewise exerting an inhibitory action. If such is the case, it should be true that eggs in sea water lacking magnesium will be more sensitive than eggs in sea water containing the normal amount of magnesium. To test this hypothesis, eggs were irradiated in artificial sea water which did not contain magnesium, and were then compared with eggs irradiated in artificial sea water containing magnesium.⁴ An irradiation time of 4 seconds was employed in order that there might be no germinal-vesicle breakdown in the eggs irradiated in the artificial sea water containing magnesium. From the results shown in Table 4 it seems clear that magnesium in the concentration in which it occurs in sea water tends to inhibit the response to ultra-violet stimulation. In the absence of magnesium the sensitivity of the eggs is increased.

In four out of the ten cases a few of the control eggs in the magnesium-free sea water which had not been irradiated showed germinal-vesicle breakdown and irregular cleavage, the percentages of breakdown being 1, 4, 13, and 16. In other words, in the absence

⁴ The formula given by Howard (1931) was used. In the magnesium-free sea water, NaCl was substituted in amount osmotically equivalent to the MgSO_4 and MgCl_2 . A pure isotonic solution of NaCl is known to have a stimulating action on the *Nereis* egg; and it is possible that the substituted NaCl , though small in amount as compared with the amount of this salt normally present in sea water, may not be entirely without effect.

of magnesium a portion of the eggs shows a spontaneous breakdown of the germinal vesicle. In view of the low pH values for the magnesium-free and Howard sea water (5.70 and 5.84, respectively), it was thought advisable to repeat the observations in more alkaline media. Eggs were placed in magnesium-free and Howard's sea water adjusted to pH 8.0-8.2 with NaOH.⁵ In each of the seven experiments performed, most of the eggs in the magnesium-free medium showed formation of two polar bodies, and some underwent irregular cleavage or lobulation (cf. Dalcq, 1924). All controls in the sea

TABLE 4
GERMINAL VESICLE BREAKDOWN FOLLOWING 4 SECONDS' IRRADIATION

No. of Expt.	Percentage of Breakdown in Magnesium-Free Sea Water	Percentage of Breakdown in Sea Water Containing Magnesium	No. of Expt.	Percentage of Breakdown in Magnesium-Free Sea Water	Percentage of Breakdown in Sea Water Containing Magnesium
1.....	99	0	6.....	100	0
2.....	100	0	7.....	97	0
3.....	100	0	8.....	45	0
4.....	99	0	9.....	78	0
5.....	97	0	10.....	100 (count uncertain)	0

water containing magnesium had intact germinal vesicles. Similar results were obtained in two repetitions of the experiment using Wheeler's formula for sea water (1910). Such sea water contains CaCO_3 and showed a pH of 7.68.

DISCUSSION

The *Nereis* egg may be considered as a delicately poised system—a system which may be thrown out of its resting state by any one of a number of stimulating agents. A stimulating agent, in impinging upon the surface of the cell, initiates a series of reactions which lead to the breakdown of the germinal vesicle; and in certain cases these reactions continue on and bring about the formation of polar bodies and irregular cleavage. An explanation of the mechanism of this series of reactions in the *Nereis* egg would presumably find wide application, for at least some of these processes take place whenever germ cells mature and cell division occurs.

This succession of changes can be inhibited or interrupted and the breakdown of the germinal vesicle thus prevented. This has been done in the present study in two ways: by the use of MgCl_2 and by prolonging the period of irradiation.

The *Nereis* egg is similar to other protoplasmic systems in that magnesium reversibly inhibits its response to a stimulating agent. Indeed, the egg may be said to undergo magnesium anesthesia. Heilbrunn (1937, chap. xxxvii) has suggested the following explanation for magnesium anesthesia. The primary effect of a stimulating agent is the release of calcium from a calcium-protein gel in the cell cortex. The free calcium then

⁵ This alkalized sea water contains no buffer, and the pH is easily changed by contact with air. Thus, samples taken from the magnesium-free and Howard sea water in which the eggs were immersed showed pH values of 6.96 and 6.84, respectively.

initiates a series of reactions (including a protoplasmic clotting), and these reactions constitute the response of the cell. With magnesium anesthesia the calcium in the cortex is replaced by magnesium, and, on stimulating, magnesium is released instead of calcium. Magnesium is much less potent than calcium in causing protoplasmic clotting; it is for this reason that the response fails to take place. That a replacement of calcium by magnesium in the cortex previous to stimulation is not necessary is shown by the result that the egg with its normal cortex can be irradiated in sea water and the response inhibited by the addition of $MgCl_2$ after irradiation has ceased. However, this does not exclude the possibility of an exchange between calcium and magnesium in the cortex following irradiation.

The importance of calcium in the process of germinal-vesicle breakdown has been suggested by several investigators (Dalcq, 1925, 1928; Hörstadius, 1923; Hobson, 1928; Pasteels, 1935); but, unfortunately, little is known of its mode of action with respect to this process. Quite possibly the inhibitory action of magnesium is due to interference with the action of calcium. Perhaps magnesium exerts an inhibitory action merely because it is less potent than calcium, as Heilbrunn suggests. If this is true, possibly a sufficient increase in the proportion of magnesium might then compensate for this difference in potency, and the response would take place. The fact that a mixture of 4 parts of $MgCl_2$ and 1 part of sea water has less effect in inhibiting germinal-vesicle breakdown than a mixture of equal parts of $MgCl_2$ and sea water would seem to lend some support to this interpretation. Pasteels (1935) has found a similar relationship for the breakdown of the germinal vesicle of the egg of *Asterias glacialis* in $MgCl_2$ -sea-water mixtures. When this egg is shed into sea water, the germinal vesicle normally breaks down. However, the percentage of breakdown is inhibited progressively as the proportion of $MgCl_2$ is increased up to a certain concentration. Further increase in the proportion of $MgCl_2$ gives less inhibition, and in pure $MgCl_2$ the breakdown is as great as in sea water.

In Pasteel's work and in the present study the breakdown is partially determined by the calcium-magnesium balance. The spontaneous breakdown of the germinal vesicle observed in sea water lacking magnesium is believed to be due in part to a disturbance of this balance. We might assume that, in a magnesium-free medium, magnesium leaves the cell (cf. Ikedo, 1937) and that free calcium, in entering to establish equilibrium, increases the proportion of free calcium. The excess calcium would then initiate the process leading to germinal-vesicle breakdown; or bound calcium may be released within the cell, and this calcium might in itself be sufficient to initiate germinal-vesicle breakdown (see Heilbrunn and Wilbur, 1937).

The inhibition of germinal-vesicle breakdown by prolonging the period of irradiation in sea water presents the interesting situation that the process producing germinal-vesicle breakdown can be inhibited even though sufficient calcium would seem to be present. Evidently, the succession of changes begun by the stimulating agent is governed by a rather delicate equilibrium. If the stimulus is of too short duration, the series of reactions may begin as indicated by a partial lifting of the membrane; but the process does not continue to the point of causing germinal-vesicle breakdown. If the irradiation is prolonged, again the series of reactions is begun, but germinal-vesicle breakdown is inhibited. Perhaps in this case an abundance of calcium inhibits the breakdown by inducing pronounced protoplasmic clotting.

SUMMARY

1. Sea-water-MgCl₂ mixtures reversibly inhibit germinal-vesicle breakdown produced by ultra-violet irradiation. The breakdown of the germinal vesicle can be inhibited by the addition of MgCl₂ subsequent to irradiation.
2. The inhibitory action of the MgCl₂ can be overcome by increasing the period of irradiation.
3. The action of MgCl₂ is antagonized by CaCl₂.
4. Germinal-vesicle breakdown and polar-body formation occur spontaneously in magnesium-free sea water.

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GROUP PROTECTION FOR EUPLANARIA DOROTOCEPHALA FROM ULTRA-VIOLET RADIATION

(One figure)

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IN SOME of his earlier experiments Allee (1928) examined the effect of water in which planarians had been living and that in which other planarians had recently disintegrated on the ability of *Euplanaria dorocephala* to withstand exposure to ultra-violet radiation. Under the conditions of those experiments such self-contaminated (conditioned) water hastened the onset and the progress of the cytolysis which is induced by ultra-violet irradiation of these worms.

The evidence at that time was recognized to be incomplete, since less contaminated water might have had a different effect. These experiments indicated that, when planarians were exposed to the action of ultra-violet rays when crowded together, retardation of cytolysis resulted or, with some worms, might be suppressed entirely. Such protective action was apparently a result of an interference with the penetration of the lethal rays, comparable with shading from visible light.

The present series of experiments have been conducted to explore further the effect of numbers present (*a*) during and (*b*) after exposure to ultra-violet radiation.

METHODS

The well-studied planarian, *E. dorocephala*, was the only experimental animal used. All the worms were collected at a spring near Cary, Illinois. About 2,000 were placed together in large enamel pans and were maintained, until needed, under conditions which have been standardized by Professor C. M. Child during his long experience with planaria in this laboratory. The planarians were kept in water from a well in the Hull Biological Laboratory basement, and the worms were irradiated in this water except as noted elsewhere. Prior to use, the worms were starved for at least a week. They were then roughly divided into lots of 20 or more of approximately the same size and were measured as they crawled over co-ordinate paper. Those needed for a given experiment were usually counted out into finger bowls the day or night before they were to be treated, and were left on the water table near the stock containers until the next morning.

The experiments were begun in March, 1937, and continued to June, 1938.¹ Results of the treatment of more than 17,000 worms are reported here, not counting those in the nonirradiated controls. The worms averaged 13.6 mm. in length, and ranged from 6 to 20 mm. In any one series of an experiment the average variation in length was 2.5 mm.; the extreme variation was never more than 4.0 mm., and this occurred only with the largest worms.

¹ Additional tests of the influence of the H-ion concentration in the medium on survival after irradiation were made during the autumn of 1938.

All singly irradiated worms were exposed to ultra-violet rays in 5 cc. of water in Syracuse dishes; crowded worms (40-50) were exposed likewise. The treatment of the less crowded, so-called "grouped" worms varied. Usually 1 cc. of water was allowed for each of the grouped individuals. Thus there were 5 worms in 5 cc. in a Syracuse dish or 20 in 20 cc. in a Petri dish. However, in certain earlier experiments, in which the effects of well water were being tested along with other solutions, as many as 120 worms were irradiated together in a Petri dish in an attempt to reduce variability and so allow for a wider comparison of results. Regardless of the numbers exposed together, one invariable rule has been followed: all worms to be used in an experiment were given exactly similar irradiation and handling in so far as we knew how to do so.

The source of ultra-violet rays was a Cooper-Hewitt lamp, which was practically new at the start of these tests. It has a horizontal "Uviarc" tube and runs on a 110-volt direct current, using 4 amperes. The planarians were exposed to the full spectrum of this air-cooled mercury-vapor arc. For more uniform dosage from day to day, we allowed the lamp to warm up for about 20 minutes before usage.

The dishes which contained the worms to be irradiated were placed on an upturned crystallization dish which was covered by a black cloth. Prior to Experiment 8 the dish was given a quarter-turn approximately each 15 seconds; thereafter the same setup was used except that it was mounted on a motor-driven turntable which made about three revolutions per minute.

Exposure to the irradiation was measured in terms of time and distance. The vertical distance from the center of the arc to the center of the base on which the dishes rested varied from 21.3 to 26.5 cm. In later experiments it was standardized at about 25.5 cm. Exposure varied from 2 to 10 minutes; it was usually from 4 to 6 minutes and was planned to bring on definitive lesions in from 1 to 3 hours.

Under the stimulus of the irradiation the worms writhe and twist; these reactions tend to result in their piling-up in the angles around the margin of the dishes. In order to insure a full exposure of each worm, one of us stirred the worms back toward the center, using a camel's-hair brush, and scattered incipient clumps.

Before starting to irradiate the worms, from four to eight series of the saltcellar type of embryological dishes were arranged near by; each series contained eleven dishes, one for the group and ten for the isolated animals. In Experiments 1-38 each contained approximately 8 cc. of the water into which the worms were to be placed. Beginning with Experiment 38, each contained a measured 5 cc. of this water.

The worms were transferred as soon as possible after irradiation into this fresh untreated water. A camel's-hair brush was used for the transfer. When various sorts of water were tested in a single experiment, a different brush was used for each type of water; and when conditions called for it, the worms were rinsed through a bath of the water in which they were to be observed. Ten irradiated worms were selected at random and placed together in one of the saltcellars; 10 more worms, which had been irradiated in the same dish (or dishes), were isolated into ten similar dishes; and so on for the various series. It is worth emphasizing that the worms were never assayed in the water in which they had been irradiated.

At all times in the following accounts, worms said to be "grouped" (G) under the ultra-violet lamp had been exposed in a Petri dish, usually 20 worms in 20 cc. of water, about 4.5 mm. deep. Those worms said to be grouped during the survival period had been placed in lots of 10 in a saltcellar embryological dish. When worms are said to be

"crowded" (Cr), they were present in such numbers during irradiation that they ~~shade~~ each other. All isolated worms (S) were placed 1 to each container.

During the period of assay, from time to time the worms were inspected under a ~~hand~~ lens or binocular dissecting microscope. The intervals between inspections depended on how rapidly disintegration was proceeding. At times it was necessary, particularly in the early stages following irradiation, to move the worms somewhat to allow critical inspection. This was accomplished with the aid of a series of glass rods, one for each type of water in the experiment. The effect of even more severe agitation was tested in Experiments 78-91, and we believe this slight disturbance had no appreciable differential effect on the time before onset of cytolysis. Frequently, but not always, the same number of isolated worms were disturbed by the glass rod that had been touched in the group, or vice versa. This careful equalization of disturbance did not seem to change the experimental results when compared with the more casual method of touching only those worms which were otherwise difficult to inspect.

Records were kept on appropriately mimeographed sheets. A system of arbitrary designation of stages was evolved early, as follows:

Stage 0. No effect to be observed.

Stage 1. Worm only generally affected; clings tightly to the substratum and appears drawn together.

Stage 2. Local effects but no lesions; margins may be rolled and swollen, usually anteriorly.

Stage 3. First lesion; usually at the anterior tip but may occur at the posterior tip, rarely in the mid-dorsal region. (If the exposure has been light, this stage may be transitory and the worm may return to an earlier stage for the time being.)

Stage 4. First lesion extends to pharynx or a second appears.

Stage 5. All dorsal integument cytolized.

Stage 6. Only a fluffy mass remains.

These stages are more or less arbitrary and represent the more easily recognized divisions of a continuous process. No attempt has been made to determine the physiological value of the intervals between the different stages. The step from stage 2 to stage 3 may have, for all we know, a different significance than that between stages 3 and 4.

The records for each set of 10 planarians, whether isolated or grouped, were added. A total stage of 30 for ten worms (that is, a mean stage of 3) was taken as the criterion for determining survival. This was done because stages 2, 3, and 4 are most clearly defined and enter chiefly into this total. Total stage 40 was almost always recorded as a check. However, if this was not reached within 12 hours, it could not be used, since experiments rarely continued longer. The records for "40" normally support the results found for "30," and little use will be made of them now beyond noting this fact (see Table 1).

The worms were treated throughout at room temperature, which ranged from 19°.4 to 28° C. during the whole series of experiments. The mean temperature was 22°.7 C., and this approaches the more usual conditions. Control series were used in the first twenty-three experiments. Through Experiment 15 and again near the end of the experimentation, these animals were irradiated *under glass*, along with those to be assayed. Later they were not irradiated but were placed in a suitable control series near the worms that had been exposed to ultra-violet rays. Neither type of control showed any cytolysis in the course of an experiment. Having established the fact that, aside from the ultra-

violet irradiation, the experimental conditions had no action, nonirradiated control series were discontinued except when room temperature was abnormal or when the worms were assayed in distilled water or some other unusual medium.

EXPERIMENTAL RESULTS

The majority of the data will be presented in the form of highly condensed summarizing tables. The amount of variation will be indicated to the initiated by the statistical probability of securing the same differences as a result of random sampling, which will be given as *P*. Probabilities have been calculated by the use of "Student's" method for

TABLE 1

A SAMPLE EXPERIMENT (NO. 13) SHOWING CYTOLYSIS STAGES OF THE INDIVIDUAL WORMS IN THE FIVE SERIES OF WHICH IT CONSISTS

Each series is considered alone; the 10 members of the group are compared with the 10 single animals, at the assay "reading" at which the singles are nearest to total stage 30. "G" grouped and "S" isolated animals. For stage values see text, page 112.

ANIMAL No.	SERIES A		SERIES B		SERIES C		SERIES D		SERIES E	
	G	S	G	S	G	S	G	S	G	S
1.....	2	3	1	3	1	3	1	3	1	3
2.....	3	3	1	3	1	3	2	4	3	3
3.....	3	3	2	3	1	3	2	3	3	3
4.....	3	3	2	3	1	3	2	3	3	3
5.....	3	3	2	3	1	3	2	3	3	3
6.....	3	3	3	3	2	3	3	3	3	4
7.....	3	3	3	3	2	3	3	3	3	4
8.....	3	3	3	3	3	3	3	3	3	3
9.....	3	3	3	3	3	3	3	3	3	2
10.....	3	3	3	3	3	3	3	3	3	3
Total stage value . . .	29	30	23	30	18	30	24	31	28	31
Minutes from start	52	52	33	33	41	41	55	55	43	43
Minutes to "30"	61	52	123	33	137	41	107	41	78	39
Minutes to "40"	155	122	221	153	250	201	190	150	153	121

data from paired experiments. When *P* equals or is less than 0.05 (five chances in a hundred of finding a like result in random sampling), the results should be given serious attention, the more so the smaller the fraction.

For many, such statistical expressions still lack meaning; hence, at the beginning we submit, in Table 1, the data from one experiment which we trust is fairly typical. Each line in all of the following tables is a summary of such data. The number of paired comparisons which are summarized is given in each case; to obtain the number of worms used, one can usually multiply this number by 20.

The effect of numbers present during irradiation.—Certain of the effects of the degree of crowding during irradiation are summarized in Table 2. In the column marked "Comparisons" the first symbol in each pair indicates the numbers present during irradiation; the second symbol shows the condition during the assay period. Thus, the first entry, CrS:GS, means that a lot of worms irradiated as a crowd and assayed while

isolated are compared with others which were irradiated as a group and likewise assayed singly. The other items in the table have been explained in the section, "Methods." The observed survival to total stage "30" in minutes is given in the fourth column, and the relative survival in percentages of time is shown in the seventh column. Statistical probabilities (*P*) are calculated for both.

In the first four sets of comparisons the worms were assayed while isolated; in the last three they were grouped. The effect of numbers present while being irradiated shows more clearly under the former condition. There is much variation in the survival of worms from the different experiments—so much so that cross comparisons are not practical. These differences result from the large number of known variables from one experiment to the next, some of which are size, condition of the planarians, time of exposure, distance from the lamp, and temperature.

TABLE 2

THE SURVIVAL OF PLANARIANS WHICH HAD BEEN IRRADIATED AS "CROWDS"
OR GROUPS OR ISOLATED, AND THEN ASSAYED ALL ISOLATED (UPPER HALF
OF TABLE) OR ALL GROUPED (LOWER HALF) DURING CYTOLYSIS

Comparison*	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	<i>P</i>	Percentage	Difference	<i>P</i>
CrS:GS.....	6	120	517:119	398	0.132	100:54	46	0.079
GS:SS.....	7	140	107:40	67	0.092	100:60	40	0.031
CrS:SS.....	6	120	517:41	476	0.090	100:24	76	0.0001
GS:SS.....	23	460	168:140	28	0.031	100:86	14	0.010
CrG:GG.....	6	120	723:172	551	0.079	100:51	49	0.132
GG:SG.....	4	80	189:80	109	0.208	100:62	38	0.105
CrG:SG.....	4	80	907:80	827	0.139	100:32	68	0.068

*"Cr," "G," and "S" as prefixes indicate irradiation in crowds, groups, and isolated, respectively. "G" and "S" suffixes show that after irradiation the worms were grouped or isolated, respectively, during cytolysis.

The variation in survival is especially high when the worms are irradiated in the crowded condition. For example, some of the worms, more than others, may remain at the top and hence get a different exposure. With "grouped" worms this type of direct protection is largely avoided, and it is impossible for those which are irradiated singly.

As a result of this variation, despite wide differences in the mean survival time in minutes, only one of the comparisons is significant when difference in minutes before onset of cytolysis ("30") is the criterion for comparison. The one significant difference (GS:SS) has the smallest mean difference of all. It is, however, the result of the largest number of comparisons, and this suggests that with more data some of the others might be significant. In cases like these it is frequently more fair to consider the difference on a percentage basis. When this is done, all the lots irradiated and assayed as isolated worms give significant differences. The apparent discrepancy between mean percentage difference and mean time difference is on account of the fact that percentage has been calculated case by case, with the longer survival evaluated as 100 per cent. If there were exceptions to the general trend, the mean percentage on the side which tends to survive longer will not be 100. For convenience in such cases this value has

been raised to 100, and the lower value on the opposite side of the compared series has been comparably corrected to maintain the same proportional mean deviation.

In common English this table shows that, when assayed singly, densely or even slightly crowded worms while being irradiated, survive better than if irradiated singly. The crowding effect was reported earlier (Allee, 1928) and is to be expected. The protection which the uncrowded group furnishes is not easily explained. What effects do these worms have on each other, either directly or by change in the water about them, that permit them to live longer after irradiation than do other worms which have been exposed similarly in all respects except that they were isolated? The fact that the grouped worms are protected is plain. The explanation, however, is not obvious. It must be remembered in this connection that these worms were not allowed to pile up during irradiation; hence the easiest explanation is untenable. Since they were assayed in fresh, nonirradiated water, mass protection from some effect of irradiation on the water—the production of ozone, for example—is not operating.

When assayed in groups, that is, with 10 worms in 5-8 cc. of water, differential effects of numbers present during irradiation are not significant. The mean differences in time to total stage 30 are even greater than before, but the variations within an experiment and from experiment to experiment are also much greater. If the trends were to continue as in these tests, further experimentation might be expected to yield significant data. Such a possibility, while interesting, does not affect the fact that with the tests as made, given equivalent differences in crowding during irradiation, significant differences are shown by the assay of isolated worms which are not revealed with the grouped planarians. This statement holds even though we do not consider the results given in the second test of the GS:SS relations, and hence limit ourselves to those which were nearly equivalent as far as numbers of worms are concerned.

The isolated state provides a more sensitive method of discovering the effects during and after irradiation than does the grouped condition. When isolated, the cytolysis of a worm affects itself only; when grouped with others in the same medium, all or a part of the activities of any individual worm preliminary to, or during, cytolysis may affect others, as well as the worm itself.

The effect of numbers present after irradiation.—In order to obtain data on the importance of numbers present during the period of assay after exposure to ultra-violet rays, all the worms being compared in any one test must be given an equal exposure. They must all be irradiated when crowded or grouped or isolated. Our experience showed more uniform results with worms irradiated as groups when the precautions previously stated were taken than when the worms were crowded together. Such group irradiation is more economical of time and perhaps more uniform than is that of the isolated worms; hence, most of our data were obtained from worms which had been exposed in groups. The results are summarized in Table 3.

We have collected much evidence on this point. These comparisons are labeled GG:GS in Table 3. The first set of tests was made in the spring of 1937. These were repeated in the following autumn to find if the same conditions held at that season. Four other repetitions were made as controls for tests of other factors. In each of the series the mean survival of planarians, equally exposed to ultra-violet, was longer if 10 worms were present together after transfer to fresh well water than if a similar number of worms were isolated each into the same amount of water which was occupied by the group. The fact that similar results have been obtained in so many different repeti-

tions in which large numbers of planarians were used in each series, combined with the relative uniformity of the percentage differences and the high statistical significance, is strong evidence of the reality of the observed difference. Enough tests were made of worms exposed singly and then assayed for survival, half grouped and half still isolated (SG:SS), to show that the group protection is present under these conditions. The variation in the exposure of individuals when they are crowded together (Cr), which has

TABLE 3
THE EFFECT OF NUMBERS PRESENT AFTER IRRADIATION UPON CYTOLYSIS

Comparison*	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	P	Percentage	Difference	P
GG:GS.....	64	1,280	148:78	70	0.021	100:78	22	0.0001
GG:GS.....	23	460	247:168	79	0.0001	100:73	27	0.0001
GG:GS.....	12	240	184:144	40	0.024	100:82	18	0.007
GG:GS.....	12	240	135:110	25	0.003	100:84	16	0.0008
GG:GS.....	19	380	64:50	14	0.005	100:82	18	0.006
GG:GS.....	25	500	169:123	46	0.038	100:86	14	0.0012
SG:SS.....	4	80	80:32	48	0.208	100:52	48	0.049
CrG:CrS.....	6	120	723:517	206	0.054	100:74	26	0.132

* In each pair of symbols the prefix shows the condition during irradiation and the suffix during assay. As before, "S" means isolated, "G" grouped, and "Cr" crowded. These symbols are used throughout and will not be explained again.

TABLE 4
THE SURVIVAL OF WORMS GROUPED OR ISOLATED THROUGHOUT THE PERIOD OF IRRADIATION AND OF ASSAY

Comparison	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	P	Percentage	Difference	P
GG:SS.....	7	140	158:40	118	0.089	100:40	60	0.0001
GG:SS.....	23	460	247:140	107	0.0001	100:60	40	0.0001

been mentioned before, accounts for the failure of worms so irradiated to show a very significant difference in survival when grouped and isolated during the period of assay; and this despite the mean difference of 206 minutes before the onset of cytolysis.

Differences in numbers present during both irradiation and assay.—The preceding tables and discussion have shown clearly our experience that, by some mechanism, grouping either during irradiation or during the period that follows before cytolysis, or during both periods, favors the survival of these planarians. As might be expected, when worms were assayed which had been grouped when irradiated and during the later period of observation and compared with those that had been isolated during both experiences, the grouped worms were found to survive decidedly longer. The pertinent experimental data have been summarized in Table 4.

Since such results are merely a verification of the work already presented, there is no call for extended discussion. All that needs to be stated is that the comparisons were, so far as we know, fairly made. The isolated worms were exposed under the same conditions (including depth of water) as were those which were grouped. The different sets of grouped and isolated worms were irradiated simultaneously in lots of 5 grouped and 5 isolated worms. Again, as always, all worms were transferred to fresh water immediately after irradiation. Table 4 shows that the observed differences are highly significant.

Groups versus isolated worms in other waters.—Well water from the Hull Laboratory well is rich in calcium and has a pH of 7.7 or more. The water from a somewhat similar

TABLE 5
SURVIVAL OF GROUPED AND ISOLATED WORMS IN SYNTHETIC POND WATER (A)
AND IN DOUBLE-DISTILLED WATER (B)

Comparison*	No. of Paired Comparisons	Total No. of Worms	Minutes to "30"	Difference	P	Percentage	Difference	P	pH
<i>A</i>									
GG:GS...	11	220	126:120	6	0.562	100:99	1	0.854	6.8
RGG:RGS...	18	360	259:261	2	0.922	100:100	0	1.000	6.8
GG:GS....	8	160	284:184	100	0.055	100:69	31	0.035	7.7
<i>B</i>									
RGG:RGS...	14	280	267:137	130	0.004	100:64	36	0.0004
RGG:RGS...	8	160	40:39	10	0.023	100:82	18	0.011
RGG:RGS...	7	140	46:31	15	0.140	100:75	25	0.053
RGG:RGS...	4	80	43:41	2	0.591	100:94	6	0.591
RGG:RGS...	9	180	454:179	275	0.110	100:69	31	0.050

* Symbols as in preceding tables except that here "R" shows that irradiation took place in the same type of water as that in which the worms were assayed.

well in the Whitman laboratories, which differs in being near relatively large animal colonies that may contaminate the ground water, is known to vary in chemical composition from time to time. In an effort to secure reproducible water, we have accordingly developed a simple synthetic pond water made by adding "reagent quality" salts to water distilled first in an aluminum still and usually redistilled in glass. Whenever distilled water is mentioned in the following pages, this double-distilled water is meant. The resulting "synthetic pond water" contained, per liter, 0.1 gm. CaCl_2 and 0.05 gm. each of MgSO_4 , K_2SO_4 , and NaNO_3 . The pH of this solution was between 6.0 and 7.0, depending on the length of time it had been exposed to the air. For some experiments this was raised to 7.7 by the addition of NaOH. Results of tests made in this water and in double-distilled water are given in Table 5.

The planarians marked GG:GS in this table were irradiated in well water and transferred to the synthetic pond water for assay. Those planarians having these

symbols preceded by "R" were irradiated in the same type of water in which they were assayed and then were transferred to fresh water of that type to find the length of their survival. Apparently it made no difference in the group-single differential in survival whether the worms were irradiated in well water or in synthetic pond water. In both sets of experiments, results from which are summarized in the first two lines of Table 5, A, there was no significant difference in survival under the conditions tested. When, however, the pH was adjusted to that of well water (7.7), the isolated worms again cytolized more rapidly than their accompanying groups, as they did also in the similar tests which were made simultaneously in well water. Some aspects of the significance of this observation will be discussed in a later section.

If one were to focus solely on the data summarized in the first line of Table 5, B, it would appear certain that the group-single differential in survival after irradiation also holds when the worms are in distilled water. Later tests, some of them with decidedly smaller numbers, were not so decisive. The grouped worms averaged a longer survival in each case, but only in one other set of comparisons are the results strikingly significant. When the whole five sets of experiments are considered together on the percentage basis, groups survived, on the average, 23 per cent longer than the accompanying isolated worms. This difference has a statistical probability of 0.0116 and must be taken seriously. As stated before, when there are so many known variables between different sets of experiments, it is fair to use the percentage difference as a basis of comparisons.

During the time taken to complete an experiment, control lots of nonirradiated worms in similar distilled water were completely unaffected, so far as cytolysis was concerned. The irradiated planarians in distilled water were much more susceptible to the treatment with ultra-violet than were worms simultaneously assayed in well water. In fact, with one dosage used (4 minutes at 26 cm.) the latter were unaffected.

There seems to be no room for doubt but that under a variety of conditions grouped worms which have been given a lethal exposure to ultra-violet irradiation survive longer than do accompanying isolated worms which have similarly and simultaneously received the same ultra-violet dosage. We are now ready to undertake an analysis of the factors underlying such group protection.

The effect of "conditioned" water.—During assay the planarians usually lived for some hours in the few cubic centimeters of water in the observation dishes. During this time the worms give off certain substances to the water, among which in distilled water are known to be: mucus; electrolytes, including calcium; carbon dioxide; and in later stages of cytolysis, proteins. Bacteria doubtless break down the organic material into decomposition products which are at present unknown. The alterations in water as a result of such living and disintegration we call "conditioning." This process will obviously proceed faster when 10 worms are placed together than if 1 worm is isolated into the same amount of water.

For an analysis of the effects of such conditioning we placed an irradiated worm in 2 cc. of water in one of the usual saltcellars and immediately or later added the irradiated worm whose survival was being followed. The two were selected so that they could easily be distinguished. If the "conditioning" worm had been lightly irradiated and was intact during most of the period of assay, the water was said to be lightly conditioned. If it was or soon became completely cytolized, the resulting water was regarded as being heavily conditioned.

In thirteen paired cases involving 260 worms in well water, the worms in lightly con-

ditioned water survived an average of 45 minutes longer than did similarly treated ones in heavily conditioned water ($P = 0.005$). On the usual percentage basis this amounts to a mean difference of 19 per cent ($P = 0.005$). This finding that the worms survived a shorter time in heavily conditioned water is in keeping with the results reported by Allee (1928). There was, however, no significant difference to be found after using seventeen series (340 worms) between those isolated into 2 cc. of fresh well water and those in lightly conditioned well water; or between fourteen series (280 worms) in which planarians were isolated, on the one hand, into 5 cc. of fresh well water and, on the other hand, into 2 cc. of such water.

These tests were repeated in distilled water without obtaining any significant differences. Evidently, in these exploratory experiments we did not reproduce the conditions that are obtained when 10 irradiated worms are placed together in 5 cc. of water.

TABLE 6

ANALYSES OF TIME TO REACH TOTAL STAGE 30 FOR PLANARIANS IRRADIATED
IN WELL WATER AND ASSAYED IN PLAIN WELL WATER AND IN
SUCH WATER WITH CALCIUM ADDED

(Experiments Involve a Total of 770 Animals)

Comparison*	No of Paired Comparisons	Minutes to "30"	Difference	P	Percentage	Difference	P
Ca:W	17	66:56	10	0.008	100:88	12	0.035
WGG:WGS	19	64:50	14	0.005	100:82	18	0.006
CaGG:CaGS	19	70:60	10	0.001	100:88	12	0.009
CaGS:WGS	18	60:49	11	0.012	100:85	15	0.034
CaGG:WGG	18	70:63	7	0.152	100:91	9	0.128
CaGG:WGS	18	70:49	21	0.0004	100:74	26	0.0006
WGG:CaGS	18	63:60	3	0.381	100:98	2	0.694

*"W," well water, "Ca," well water with increased calcium.

The effect of calcium.—One of the decided differences between distilled and well water is the absence of calcium from the former. Earlier work (Oesting and Allee, 1935) had shown that calcium retards cytolysis of marine *Procerodes* placed in fresh water and of *Euplanaria* when in distilled water (Buchanan, 1935). This similar deficiency of calcium may be the reason why the planarians in distilled water survived the effects of ultra-violet irradiation for so much shorter a time than did those simultaneously and equally exposed and assayed in well water.

Well water with increased calcium: The well water which was the basic medium for much of this work contained between 40 and 50 mg. of calcium per liter. Enough CaCl₂ was added to make a M/120 solution in well water. The planarian worms were irradiated in well water and then for the assay period were transferred to fresh well water or to the water with calcium added. Results are summarized in Table 6.

As one reads down Table 6, it is evident that in the seventeen cases in which comparisons are entirely fair, the grouped and isolated worms, considered together, survived 10 minutes longer ($P = 0.008$) in well water to which calcium had been added than they did in well water alone. In both media the usual group-single differential showed

significant differences. The isolated worms lived longer in the stronger calcium solution, while there was an insignificant difference with grouped worms. This verifies findings already reported that the assays made on isolated worms are more sensitive than those made with grouped planarians.

In keeping with all these results, the grouped worms in the well water plus calcium survived better than did the isolated ones in well water. However, the added protection furnished the group in well water caused such worms to live slightly but insignificantly longer than did isolated individuals in the calcium-rich water.

TABLE 7
SHOWING SURVIVAL AFTER IRRADIATION WHEN ASSAYED IN SEVERAL
DILUTIONS OF CaCl_2
(Specific Conductivity in Mhos $\times 10^{-6}$)

Dilution	Comparison	No. of Paired Comparisons	Minutes to "30"	Difference	P	Percentage	Difference	P	Specific Conductivity
M 0.0069 . . .	WGG:WGS	8	49:39	10	0.023	100:82	18	0.011	1.6
	CaGG:CaGS	8	386:201	185	0.047	100:68	32	0.020	2,209
	CaGG:WGG	8	386:49	337	0.009	100:21	79	0.0001
	CaGS:WGS	8	201:39	162	0.003	100:23	77	0.0001
	Ca:W	8	294:44	250	0.005	100:30	70	0.0001
M 0.001	WGG:WGS	7	46:31	15	0.140	100:75	25	0.053
	CaGG:CaGS	12	445:386	59	0.162	100:71	29	0.007	252 3
	CaGG:WGG	9	442:39	403	0.027	100:12	88	0.015
	CaGS:WGS	8	497:29	468	0.041	100:11	89	0.0001
	Ca:W	7	526:38	488	0.061	100:13	87	0.0001
M 0.00001 . . .	WGG:WGS	4	43:41	2	0.591	100:94	6	0.591
	CaGG:CaGS	4	103:87	16	0.127	100:80	20	0.068	4 5
	CaGG:WGG	4	103:43	60	0.115	100:50	50	0.014
	CaGS:WGS	4	87:41	46	0.208	100:59	41	0.032
	Ca:W	4	95:42	53	0.154	100:53	47	0.012
M 0.000001 . . .	WGG:WGS	9	454:179	275	0.110	100:69	31	0.050
	CaGG:CaGS	9	697:250	447	0.007	100:39	61	0.0001	1.5*
	CaGG:WGG	9	697:454	244	0.047	100:56	44	0.005
	CaGS:WGS	9	250:179	71	0.148	100:79	21	0.007
	Ca:W	9	474:317	157	0.015	100:60	40	0.002

* Distilled water at this time tested 1.4×10^{-6} mhos.

Such results showing the importance of calcium in survival of irradiated planarians led to an attempt to find the minimum amount which could be added to secure protection. Tests were made in solutions of CaCl_2 in distilled water of the following strengths: 0.0069 M, 0.001 M, 0.00001 M, and 0.000001 M. All exposures to ultra-violet radiation were made in double-distilled water. Irradiated controls were tested simultaneously in the distilled water, and nonirradiated controls were also used. These latter showed some lesions in the weaker dilutions but never approximated the rate of cytolysis of the animals treated with ultra-violet. Summaries of these tests are given in Table 7.

For reasons already noted, when the known variables are as many and as large as in

these experiments, the percentage comparisons are fairer than a study of time differences. Focusing, then, on the percentage columns, one notes that the group versus isolated comparisons in distilled water do not always yield significant results in each separate series; when, however, the whole experience with distilled water is considered together, there is no doubt but that the worms assayed in groups live longer after exposure to ultra-violet than if they are isolated.

Similar comparisons in the various dilutions of CaCl_2 also show significantly longer survival if assayed in groups than if isolated. All but one of the series tested is clearly significant, and there is no doubt of the difference when all the series are considered together. In all comparisons, worms assayed in various concentrations of CaCl_2 solutions survived longer than did similar worms in distilled water. This holds true even with group comparisons, which are less critical in many ways than are those of isolated worms. As would be expected, with both separate parts significant, the combined comparisons are highly so.

It is worth noting that a solution of $M\ 0.000001\ \text{CaCl}_2$ with a conductivity of only 1.5×10^{-6} mhos still gave decidedly longer survival after irradiation than did distilled water with a slightly less conductivity (1.4×10^{-6}). Buchanan (1935) found that similarly extreme dilutions of calcium protected *E. dorotocephala* when placed in hypotonic water without having been irradiated. In our experience, after irradiation cytolysis was delayed by exposure either to hypotonic or to hypertonic solutions of CaCl_2 .

Parallel experiments with similar concentrations of MgCl_2 demonstrated that this salt also delays cytolysis after irradiation with ultra-violet; the effect, however, is not so marked as with similar concentrations of CaCl_2 . This relationship can be stated without giving even the abbreviated summaries we have been using. Both $M\ 0.001$ and $M\ 0.00001$ solutions of CaCl_2 delayed cytolysis significantly longer than did the same strength of MgCl_2 . With $M\ 0.000001$ the worms survived 21 per cent longer in MgCl_2 than in distilled water ($P = 0.128$), while they survived 40 per cent longer in CaCl_2 ($P = 0.002$).

Other experiments verified these results. With artificial pond water it is possible to use MgCl_2 in place of CaCl_2 . The solution then contained, per liter of distilled water, 0.1 gm. MgCl_2 , 0.05 gm. each of MgSO_4 , K_2SO_4 and NaNO_3 . At times worms were entirely normal in appearance after 4.5 days in such water, and in no case was a nonirradiated control in this calcium-free water affected during the progress of an experiment. After irradiation grouped and isolated worms survived significantly longer in well water and in the regular synthetic pond water than they did in calcium-free synthetic pond water.

These observations, especially the results with $M\ 0.000001\ \text{CaCl}_2$, suggest that, if the group gives off more calcium into the assay water than do the isolated worms, this may be the mechanism of the group-single differential.

Proportional volumes of well water.—In order to test the effect of proportional, rather than equal, volumes on survival after irradiation, the planarians, exposed in lots of 20 per Petri dish in well water as usual, were then placed in saltcellar embryological dishes, 10 together in 10 cc. of fresh well water and 1 each in ten dishes in 1 cc. of similar water. The volume of water was thus proportional to the number of worms being tested; the depth was unequal, but the exposed surface was approximately the same for grouped and for each isolated worm. To avoid excessive evaporation, all were kept in moist chambers. The results are summarized in Table 8, A. With the volume of water during assay proportional to the number of worms present and arranged, so that there was 1 cc. of water

for each worm whether grouped or isolated, the worms still lived significantly longer if assayed when grouped. The difference in minutes is 178 with $P = 0.019$.

As a further refinement, after irradiation as before, 9 worms were put into the usual embryological dishes in 9 cc. of water, and 9 others were isolated in 1 cc. of water, each in a narrow shell vial. The depths were practically the same, although the exposed surface was much less in the shell vials. Under these conditions, as shown in Table 8, *B*, the usual group-single differential was destroyed; and while there was no significant difference, the isolated animals averaged longer survival.

These results, taken together, indicate that in the tests as usually made, where volume, surface area, and depth were all equal regardless of numbers present, there may have been some volatile substance (carbon dioxide, for example) which accumulated more rapidly among the grouped worms and retarded their cytotoxicity.

TABLE 8

SURVIVAL IN PROPORTIONAL VOLUMES OF WATER: A, WITH UNEQUAL DEPTH AND EQUAL SURFACE; B, WITH DEPTH EQUAL AND SURFACE UNEQUAL
(A, 340 Worms; B, 280 Worms)

Comparison	No of Paired Comparisons	Minutes to "30"	Difference	<i>P</i>	Percentage	Difference	<i>P</i>
GG:GS	<i>A</i>						
	17	474:296	178	0.019	100:68	32	0.0002
	<i>B</i>						
GG:GS	14	218:237	19	0.336	95:100	5	0.496

The effect of agitation on cytotoxicity.—During inspection of the planarians for possible effects of irradiation, it was sometimes necessary to move the worm slightly in order to secure adequate inspection. This was more likely to be true for grouped worms. For this purpose we used a rounded glass rod and handled the worms as gently as possible. Sometimes when one or more worms of the grouped lot were touched, a similar number of the accompanying isolated series would be similarly treated. Often this was not done. It is possible that such mechanical stimulation may have hastened the onset and progress of cytotoxicity. If so, the grouped worms should have been more affected, and this would have worked against the observed results.

In order to test the effectiveness of this factor, we set up parallel series of worms each with the usual groups of 10 and 10 accompanying isolated individuals. One such set was left undisturbed until near "30," and then the reading was taken with the minimum disturbance. In another series all animals were briskly stirred with a camel's-hair brush about five times between each reading until "30" was reached. An analysis of the results obtained appears in Table 9.

As usual, the group-single differential survival shows in the unagitated worms, while

in the agitated ones it has entirely disappeared. The situation, however, is not quite this simple, for the disturbed groups survived a significantly shorter time than did those which were undisturbed. The isolated worms, on the other hand, show no significant difference. When averaged together, the agitated grouped and isolated worms survived a significantly shorter time than the unagitated ones.

The fact that the isolated worms are not significantly affected by the disturbance given them indicates that the results are not caused by direct action of the agitation on the worms themselves. In the usual assays any disturbance of the worms would tend to break down the observed group-single differential survival; and since, in actual practice, group worms were touched more than isolated ones, the observed group effect, as demonstrated, for example, in Table 3, cannot have been a result of differential stirring while making the observations.

This evidence indicates that the agitation may disperse some material from the water around the grouped worms which otherwise would delay cytolysis. Calcium would not

TABLE 9
THE EFFECT OF AGITATION ON RATE OF CYTOLYSIS
(Each Comparison Involves 12 Paired Cases and a Total of 240 Worms)

Comparison*		Minutes to "30"	Difference	P	Percentage	Difference	P
Unag.	GG:GS.	135:110	25	0.003	100:84	16	0.001
Ag.	GG:GS.	101:101	0	1.000	99:100	1	0.922
Groups	Unag:Ag.	135:101	34	0.005	100:77	23	0.002
Isolated	Unag:Ag.	110:101	9	0.162	100:91	9	0.009
Combined	Unag:Ag.	123:101	22	0.012	100:83	17	0.005

* "Ag," agitated; "Unag.," unagitated.

be much affected by such treatment, while carbon dioxide would escape readily from the agitated water.

The effect of pH on rate of cytolysis.—The experiments here reported grew out of the observation that the survival of worms in regular synthetic pond water was much longer than in well water. Among the known variants are: The synthetic pond water is an unbuffered water with approximately half the calcium content found in well water. Also, the pH of the well water is much higher, ranging from 7.7 to 8.2. The synthetic pond water, on the other hand, has a pH comparable with distilled water; in the spring of 1938 both were about pH 6.0 when fresh and approached neutrality on standing.

The pH was changed, as needed, by adding a few drops of 0.1N NaOH or HCl. Carbonate-free well water was obtained by acidifying well water with concentrated HCl below pH 4 and then allowing a stream of compressed air to bubble through for about 18 hours; after this the pH was raised to the desired level with NaOH. Hellige indicator and standards were used to determine pH.

The treatment of animals and the experimental setup were as usual unless mentioned otherwise. The worms were irradiated in synthetic pond water or in well water, depending on which was basic for the given experiment. After rinsing with fresh water of the type to be used in the assay, they were transferred to the assay dishes. With three exceptions (tests 1, 2, and 5 of Table 10, B) all animals were assayed singly in series of 10

isolated worms. In the case of the exceptions, groups and singles have been considered together. At the time of filling the assay dishes, one extra dish for each medium was also filled with 5 cc. of that water, and 0.5 cc. of Hellige indicator was added. In this way the pH, together with any drift, could be read at any time during the experiment provided the light was suitable.

In the experiments made during the autumn of 1938 the pH of unbuffered waters shifted slightly, if at all. Only acidified well water displayed this phenomenon to any

TABLE 10
THE EFFECT OF pH ON SURVIVAL AFTER IRRADIATION
(Specific Conductance Is Given in Mhos $\times 10^{-4}$)

Water 1:2	Mean pH 1:2	No. of Paired* Com- parisons	Minutes to "30"	Dif- fer- ence	P	Percent- age	Dif- fer- ence	P	Specific Conduct- ance† 1:2
A, Comparisons within One Medium between pH below and above 7.0									
Well:Well.....	5.8:7.6	3	194:127	67	0.105	100:68	32	0.039	3.3:2.8
	6.3:8.0	8	309:162	147	0.013	100:54	46	0.001	3.2:2.8
SP:SP.....	6.2:8.3	12	160:195	26	0.295	95:100	5	0.498
	6.7:8.0	12	166:165	1	0.922	100:99	1	0.627	2.9:2.9
CO ₃ -free:CO ₃ -free...	6.2:7.8	11	136:175	39	0.037	85:100	15	0.009	equal
B, Comparisons between Two Media of Different or Same pH									
SP:Well.....	6.8-:7.5+	6	377:110	267	0.017	100:35	65	0.000
	6.8-:7.5+	11	124:86	38	0.001	100:72	28	0.031
	6.7:8.0	4	134:80	54	0.068	100:63	37	0.012	2.9:2.8
	6.4:6.5	18	144:178	34	0.061	88:100	12	0.009	2.9:3.2
CO ₃ -free:Well.....	6.2:7.7	5	229:146	83	0.027	100:66	34	0.023	6.0:2.8
	6.3:6.1	6	218:150	68	0.021	100:70	30	0.004	6.8:3.3
	6.3:6.3	7	167:182	15	0.047	91:100	9	0.041	8.0:8.8
	5.8:5.7	12	156:147	9	0.295	100:98	2	0.628	9.0:8.9

* To find the number of worms used in each test, multiply the number of paired comparisons by 20, except in the first, second, and fifth test under B, where 40 worms have been used in each paired comparison.

† Specific conductance has been deduced for most of these waters by tests on similar water of similar pH. In the last three tests, however, the specific conductance is that of the same lot of water used in the survival assay.

marked extent. (There is evidence of reduced ammonia fumes in the air, as compared with the spring of 1938.) Acidified well water showed a shift in pH of 0.1 unit per 14 or 15 minutes. Unmodified well water, on standing, had a slow pH drift up to pH 8.3 at the rate of 0.1 unit per 34-60 minutes. Carbonate-free well water initially below pH 6.4 showed a mean rise at the rate of 0.1 unit per 75 minutes (extremes 17.5 to no rise at all). Synthetic pond water of low pH gave irregular results; it showed a rise, if at all, of 0.1 unit in an average of 113 minutes (extremes, 18 minutes to no change in 5 hours).

Our experience regarding the effect of pH on survival after irradiation is summarized in Table 10. The experimental results indicate:

1. Isolated irradiated planarians survive significantly longer in well water below

neutrality. If, however, the carbonates are first removed from this water, they then survive significantly longer in water above pH 7.0.

2. In synthetic pond water (which lacks carbonates) there is no difference in the survival of the isolated worms in the water above and below pH 7.0.

3. The planarians survive significantly longer in synthetic pond water at its usual pH than in unmodified well water, which usually has a pH approximately a whole unit higher.

4. When, however, the well water is acidified below neutrality, survival of the worms is increased over that occurring in synthetic pond water.

5. Animals tested in carbonate-free well water and in well water of low pH survive significantly longer in the former. This is, however, readily explained by the large increase in the electrolytic content of the carbonate-free water. After adjustment of this factor is made, it is found that—

6. When the worms were tested in carbonate-free well water, as contrasted with ordinary well water brought to the same pH, and in which the electrolytic content slightly exceeded that in the carbonate-free well water, the opposite condition obtained; i.e., the animals survived significantly longer in the ordinary well water.

7. When both the pH and the electrolytic content were practically equalized, there was no difference in survival time following irradiation.

The situation revealed by these experiments is not simple. Dismissing variation due to the physiological condition of the planarians, to differences in irradiation, and to temperature, at least four factors may be acting in the experiments summarized in Table 10 to affect the length of survival of the irradiated worms. These are: H-ion concentration per se, carbon dioxide concentration, total electrolytes present, and, perhaps, the ionic composition of the different waters used. The observed relations are schematically summarized in Table 11.

The first comparison in Table 11, *A*, taken alone, indicates that longer survival depends on a lower pH, more free CO₂, and more total electrolytes. The second comparison tends to rule out the importance of pH differences, at least in synthetic pond water; in fact, only in carbonate-free well water does pH seem to be a determining factor, and then longer survival was found in the more alkaline water (cf. Buchanan, 1930).

The first two comparisons in Table 11, *B*, indicate that the longer survival in synthetic pond water, as compared with well water, is a result of differences in concentration of total electrolytes. If these data represent the facts in the case, a relatively small difference in total electrolytes is effective. The other comparisons strengthen this conclusion concerning the importance of electrolytic concentration.

As will be shown in the next section, in waters having an electrolytic concentration such as is found in synthetic pond water, conductivity tests do not reveal any increase in total electrolytes when 100 planarians have lived in 100 cc. of calcium-free synthetic pond water for 4 hours. Since we are primarily concerned with the observed differential survival of grouped and isolated planarians following irradiation, these experiments raise immediately a question concerning the effect of groups on pH. With well water, which was the best buffered water we used, at total stage 30 we found that the water with about ten series of isolated worms had a pH of 7.8, while the accompanying groups were in water of pH 7.7 (difference, 0.1; $P = 0.003$). In nine other series at total stage 40 the same values were pH 7.7 and 7.3 (difference, 0.4; $P = 0.0001$). As far as they go, these differences would make for longer survival on the part of the grouped worms, primarily

because of an indicated increase in free carbon dioxide. There was little or no carbonate to be broken down in the synthetic pond water. Hence we should not expect to find a group-single differential so readily in this water; as shown in Table 5, this expectation was realized.

Effect of irradiation on permeability.—There is evidence that irradiation with ultra-violet increases permeability of living cells (Heilbrunn and Mazia, 1936). It is important in developing an explanation of the group protection from exposure to ultra-violet radiation to know the effect of the treatment on permeability of planarian worms. This was tested by measuring the change in total electrolytes as the worms remained in various

TABLE 11

A SCHEMATIC SIMPLIFIED SUMMARY OF THE DATA PRESENTED IN TABLE 10
(The Apparently Important Factor or Factors Are Italicized or Shown in Bold Face)

Water 1:2	pH 1:2	Free CO ₂ 1:2	Electrolytic Content 1:2	Ionic Constitution	Longer Survival
	<i>A</i>				
Well:Well	7-:7+	<i>more:less</i>	<i>more:less</i>	<i>approx. same</i>	1
SP:SP	7-:7+	<i>approx. same</i>	<i>approx. same</i>	<i>approx. same</i>	equal
CO ₂ -free:CO ₂ -free...	7-:7+	<i>approx. same</i>	<i>approx. same</i>	<i>approx. same</i>	2
	<i>B</i>				
SP:Well	7-:7+	<i>less:more</i>	<i>more:less</i>	<i>different*</i>	1
SP:Well	<i>approx. same</i>	<i>less:more</i>	<i>less:more</i>	<i>different</i>	2
CO ₂ -free:Well	7-:7+	<i>less:more</i>	<i>more:less</i>	<i>different</i>	1
CO ₂ -free:Well	<i>approx. same</i>	<i>less:more</i>	<i>more:less</i>	<i>different</i>	1
CO ₂ -free:Well	<i>approx. same</i>	<i>less:more</i>	<i>less:more</i>	<i>different</i>	2
CO ₂ -free:Well	<i>approx. same</i>	<i>less:more</i>	<i>approx. same</i>	<i>different</i>	equal

* The difference between "SP" and "Well" is great, both quantitatively and qualitatively. Except for the absence of carbonates in one, the two types of well water differ only in quantity of certain ions.

waters after irradiation, as indicated by measurements of conductivity made by standard methods.

The usual calcium-free artificial water (p. 121) had a conductivity of 325×10^{-6} mhos, while a similar amount of the same water in 100 cc. of which 100 nonirradiated planarians had stood for 5 hours had a conductivity of 319×10^{-6} . In a parallel test with double-distilled water the blank tested 2.9×10^{-6} , and the water with worms showed 14.5×10^{-6} . Water collected from seven groups of 10 irradiated worms and from 20 single animals, prior to "30," showed no increase in electrolytic content over that found in the blank. Apparently neither irradiated nor normal worms give off electrolytes into artificial pond water; more comprehensive tests are given in Table 12. Two lots of 100 cc. solution—one with, and the other without, 100 normal worms—were left in 150 cc. Erlenmeyer flasks for 4 hours. This was a common length of time for irradiated worms to have begun cytolysis. At that time conductivity measurements were made. Again the results show that in full-strength calcium-free synthetic pond water there is

no gain in conductivity after planarians have lived in the water for 4 hours; rather there appears to be a small loss, as though something had been taken from the water. With increasing dilution there is a fairly steady increase in conductivity.

As a result of these tests, all further experiments on these points were made in double-distilled water.

Preliminary experiments indicated that the irradiated worms gave off electrolytes more rapidly than did nonirradiated ones. Accordingly, four experiments were made to follow the progressive loss of electrolytes into double-distilled water by lots of irradiated and untreated (control) worms. In Experiment C7, 500 worms were placed in 1,000 cc.; in C8, 200 worms were used per 300 cc.; in C9 and C10, 100 large worms were put in 100 cc. The worms in C9 were large (16–18 mm. long) and were sufficiently resistant so that they did not cytolysis according to the usual pattern following ultra-violet irradiation; the disintegration which finally took place was more like that found in the controls

TABLE 12

TESTS OF THE LOSS OF ELECTROLYTES FROM NORMAL WORMS
INTO CALCIUM-FREE ARTIFICIAL POND WATER OF FULL
STRENGTH AND OF VARIOUS DILUTIONS

Percentage of Pond Water	Blank 10 ⁻⁶ Mhos	Experimental 10 ⁻⁶ Mhos	Gain in Mhos
100.	333 8	331 6
57 3	204 9	201.4
33.3	119 1	123 2	4 1
16 6	63 9	66 4	2 6
8 3	34.8	40 6	6 8
0 0	3.31	11 6	8 2

as a result of exposure to hypotonic water. In C10 the dosage was increased so that the irradiated worms showed the usual effects.

Except in Experiment C9, where it will be recalled that the dosage was insufficient to bring about cytolysis from ultra-violet irradiation, the loss of electrolytes is initially greatest in the irradiated worms. This increased rate of loss starts before there is any visible sign of an actual lesion. With the beginning of observable lesions, the rise in conductivity, that is, the loss of electrolytes, is greatly increased. Even in Experiment C9 the tendency toward greater loss by irradiated worms during the first few hours is noteworthy; and since it is a trend covering some five readings, it probably indicates an initial effect of the irradiation from which the worms later recovered.

Is calcium the protective factor?—Results summarized in an earlier section demonstrate that CaCl_2 , even in extremely dilute solution (0.000001 M), delays the onset of cytolysis following ultra-violet irradiation. The work given in the last section shows that after such irradiation, tissues of planarian worms, on being placed in distilled water, are more permeable to contained electrolytes than are nonirradiated controls. The loss of electrolytes becomes much more rapid when actual lesions appear. These observations raise an obvious question: Is the accumulation of calcium around the grouped animals the factor which produces the observed group protection?

Unless otherwise noted, the results have been obtained by a titrimetric method which

is a combination of the reagents and procedures in several calcium oxalate methods of analysis (Peters and Van Slyke, 1932; Van Slyke and Sendroy, 1929; American Public Health Association, 1933).

Specifically, 500 cc. of the water to be analyzed was boiled down in a flask to about 75-100 cc. At some point during the boiling, 1 cc. of concentrated HCl was added to break down the carbonates. When the boiling was completed, the flame was lowered to maintain a simmer. Twelve drops of bromcresol green indicator (0.016 per cent solution) were added; then 2 cc. of ammonium oxalate; and, lastly, enough 1:1 NH_4OH to change the solution from yellow to a clear blue. This gave a pH of about 5 or more. At this pH calcium oxalate is precipitated. If indicated, more ammonium oxalate may be

TABLE 13

PROGRESSIVE LOSS OF ELECTROLYTES INTO DISTILLED WATER BY NORMAL AND BY IRRADIATED WORMS (TIME AT WHICH THE FIRST CASE OF CYTOLYSIS WAS OBSERVED MARKED BY ASTERISK)
(All Values in Mhos $\times 10^{-6}$)

TIME IN HOURS	EXPT. C7		EXPT. C8		EXPT. C9		EXPT. C10	
	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
0 0.....	5 2	5.2	3.9	3 9	3 9	3.9	5 4	5 4
0.1-0.4.....	5.3	5.7	4 4	5 2	4 9	6 7	5 1	8.9
0.75-1.0.....	5 8	6 0	5.6	7.3*	5.3	7.5
1.3-1.5.....	6.5	7 0	6.1	8.2	7.1	7.5	6 2	8.7*
1.6-2.0.....	7 0	7.7	6 4	8.9	7.2	8.5	6.8	10.3
2.5-3.0.....	7.8	9.5*	8 8	8.9	8.3	14 5
3.8-4.5.....	9.7*	12.6	9 8*	18.1	10.3	10.8
5.0.....	10.6	14.7	12.2	25 1
6 0.....	12.5	17 3	13 8	13 7	13.5	30.6
6.8-7.0.....	14.2	20.1	13.9	24 1	15.3	13.7
8.0.....	16 9	21.7	15.7	14.3
18 8-23.2.....	36 3	36.0	41.8	39.5	44.1*	34.7*	38.8*	53.6
30.....	40.1	37.8	51 8	52.2
44.5-45.5.....	65.6	64.4	59.8	69.6	75.4	60.0

added. The mixture was then shaken occasionally and allowed to simmer for 10-15 minutes. When the precipitate settled readily after shaking, it was filtered through a No. 40 Whatman ashless filter. The filtrate was tested by addition of ammonium oxalate to assure the complete removal of calcium. About 50 cc. of a 2 per cent solution of NH_4OH was heated and used in four portions to wash down the precipitate in the filter paper. Complete washing-out of all excess ammonium oxalate is of great importance.

Fifty cubic centimeters of normal H_2SO_4 were heated to boiling and poured in portions over the filter paper containing the precipitated calcium oxalate while this was spread over the side of a beaker. The solution of oxalic acid so formed was transferred to a 125-cc. Erlenmeyer flask, heated to boiling, and titrated hot with N 0.05 (or N 0.01) potassium permanganate. Analysis of the amount of calcium in synthetic pond water showed that the titrimetric method outlined above gave 22.2, 21.2 (mean 21.7) mg. per liter. The gasometric method of Van Slyke and Sendroy gave 22.6, 19.9, 22.7, and 19.5 (mean 21.2) mg.; and a titrimetric modification of this method gave 23.3, 21.75,

and 21.50 (mean 22.3) mg. The calculated amount of calcium in this water was 21.9 mg. per liter. A test solution containing by weight 4.007 mg. of calcium per liter yielded 4.01, 4.11, 4.29, 5.12, 4.60, and 4.57 (mean 4.48) mg. per liter. One containing about 0.04 mg. of calcium per liter yielded 0.96, 0.84, and 0.65 (mean 0.82) mg. The previous test in distilled water had given 0.126 mg. per liter. Apparently the washing-off of the ammonium oxalate from the precipitated calcium oxalate had been incomplete. This error is hard to avoid.

Within a range of from about 22 to 4 mg. per liter this titration method is fairly reliable.

Tests of the amount of calcium given off by planarians into distilled water were then made. Five hundred worms irradiated 2 minutes at 26.6 cm. were placed in 500 cc. of double-distilled water. Eight hours later they averaged one lesion each (equivalent of total stage 30 of the usual assay test⁹); the distilled water blank showed 0.066 mg. for the 500-cc. sample; and the water decanted from the cytolyzing worms gave 0.50 mg. per 500 cc. Another lot of 250 worms were irradiated and placed in 250 cc. of double-distilled water. They cytolyzed to mean stage 3 in 10 hours. The accompanying distilled water blank showed 0.056 mg. of calcium, and water decanted from the worms contained 0.225 mg. per 250 cc.

From these data it appears that the amount of calcium lost into distilled water by 500 cytolyzing worms in 500 cc. of water is on the order of 1 mg. per 1,000 worms per liter, a value which lies below the tested limit of the method used. Considering the determination as valid, this would amount to about 0.001 of a milligram per worm.

The method used is relatively reliable at a level of 4 mg. calcium per liter. It is perhaps noteworthy that the solution from the analysis of worm water showed a faint cloudiness after the intended precipitation of the calcium as oxalate, a condition which is lacking in the various blanks. Hence, we may not have gotten quite all the calcium that was present.

It is evident that we were unable to find or develop methods of analysis which would allow us directly to test the hypothesis that the observed group protection results from the greater addition of calcium by the group. If we accept the results obtained at their face value, by calculation we would expect that for the group at total stage 30 there would be approximately 0.01 mg. of calcium in 5 cc. of water, while the water around the associated isolated planarians would contain only 0.001 mg.

From direct experimentation we know that 0.000001 M CaCl_2 does retard the onset and progress of cytolysis following irradiation. This amounts to an increase of 0.04 mg. of calcium per liter, or to 0.0002 per 5 cc.; hence it would be expected that the calculated increase of 0.01 mg. in 5 cc. of distilled water could result in a decreased rate of cytolysis as compared with water containing one-tenth that amount.

Any general theory that the observed group protection in well water and alkaline synthetic pond water is a result of calcium leached from the grouped worms runs against the observation that there is no measurable change in the electrolytic content of such water after worm cytolysis. There are other objections to considering protection as a result of increased calcium content to be the entire explanation of the observed phenomena. These have already been presented and will be reviewed later. Now it is necessary to consider another differential factor.

The effect of carbon dioxide on the rate of cytolysis.—In order to test the effect of increased carbon dioxide on the onset and progress of cytolysis of irradiated planarians, a

number of experiments were set up as follows: Six series of eleven saltcellars were arranged, each containing the usual 5 cc. of water. Well water was used throughout. A twelfth dish was added which contained the same amount of water plus Hellige indicator. Each series was placed in a covered crystallization dish, three of which were charged with properly washed carbon dioxide from a Kipp generator until the indicator dish showed a pH of 6.0. The worms were irradiated as usual in lots of 20 per Petri dish. Two such lots, as nearly equal in size and other relations as possible, were distributed one half of each lot to an experimental and the other half to a control series. The six series gave three paired comparisons for each experiment.

The three experimental chambers were recharged with the gas after the animals were set out, and then again at or near total stage 30, for the obvious reason that carbon dioxide readily escapes if exposed to ordinary atmosphere. Appropriate controls showed that nonirradiated worms were not visibly affected by such carbonated water. The results from a number of paired series, each of which contained in all 500 worms are summarized in Table 14.

TABLE 14
THE EFFECT OF CARBON DIOXIDE ON THE RATE OF CYTOLYSIS
(Each Comparison Based on 25 Paired Series)

Comparison*	Minutes to "30"	Difference	P	Percentage	Difference	P
WGG:WGS	169:123	46	0.038	100:86	14	0.0012
CDGG:CDGS	225:195	30	0.017	100:87	13	0.0014
CDGG:WGG..	225:169	56	0.025	100:79	21	0.0018
CDGS:WGS	195:123	72	0.003	100:77	23	0.0002
CD:W . . .	210:146	64	0.006	100:78	22	0.0002

* "W," well water control; "CD," carbon dioxide treated water.

The group-single differential appears significantly in both the well water controls and the carbon dioxide experimental series. The worms treated with carbon dioxide, both grouped and isolated, survived significantly longer than did similar ones placed in well water.

DISCUSSION

In general terms our knowledge concerning the effect of numbers on different biological processes can be summarized in the two curves shown in Figure 1, in which distance above the base line indicates the speed of some biological process, or group of processes, and distance to the right shows increasing population density.

Some aspects of mass relations show no effect of undercrowding (Fig. 1, A); such biological processes are typically retarded with increasing numbers. The experience of Pearl and Parker (1922) with effects of numbers of pairs of *Drosophila* on the rate of egg-laying per female is a well-known case in point. Other phases of group relations are summarized in Figure 1, B (cf. Allee and Bowen, 1932). In these there is a maximum increase in some physiological relationship at some distance above the minimal numbers possible, usually well before the maximum population is reached.

As Allee (1938) says, it would be better perhaps, in so far as this is possible, to view population effects in terms of these descriptive curves rather than as harmful or bene-

ficial results. Such terms, if used to interpret survival values only, are relatively objective; they are, however, somewhat ambiguous and can be interpreted in a more anthropomorphic manner.

The effects of undercrowding, shown by the left-hand limb of the curve in Figure 1, *B*, have been reported for a wide variety of organisms and conditions. Many of these cases have been reviewed elsewhere (Allee, 1931, 1934, 1938), and no attempt will be made to list or discuss other pertinent cases which are to be found in the widely scattered and growing literature on the subject. It suffices our present purpose to say that in the present state of knowledge a first concern in undertaking a study in population physiology is to find whether the phenomena under investigation belong to those which can be summarized by curves related to Figure 1, *A*, or to Figure 1, *B*. After settling this point, a second and more difficult problem is concerned with the analysis of the factors involved in producing the observed results.

The case reported in the early sections of this paper, that of group protection for *E. dorotocephala* during and after exposure to ultra-violet radiation, belongs obviously

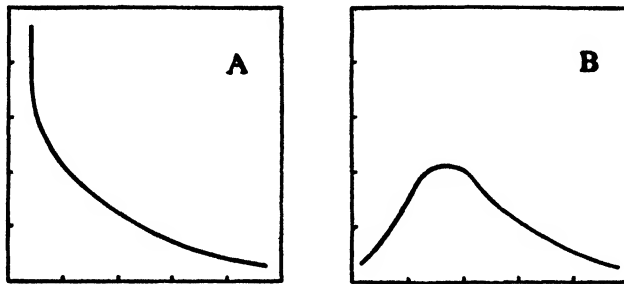


FIG. 1. Curves summarizing the effects of numbers present on biological processes

to the *B*-type of curve. At the dosages used, few of the planarians survived indefinitely. They were purposely irradiated sufficiently so that such would be the case. With a shorter exposure, which was sometimes unintentionally given, we could have found a dosage at which all the isolated worms would be killed off while many, sometimes the majority, of the grouped worms would recover. Having established the existence of undercrowding under these conditions, we then proceeded to the harder task of trying to find the mechanism or mechanisms involved.

It is pertinent in this connection to note again that immediately after irradiation the planarians were transferred to fresh, that is to say, nonirradiated, water to await cytolysis. This transfer after irradiation was made by changing one or a few worms to the group and then isolating a similar number.

We know that being left in the water in which they were irradiated hastens the beginning of cytolysis. In test series half were transferred immediately as usual, and half after a delay of 5 minutes. Both grouped and isolated worms cytolized more rapidly if the transfer was delayed. In the given instances they survived (to total stage 30) 26 per cent shorter time with $P = 0.042$.

In the usual group-single tests there was more irradiated water introduced into the water about the group than into that about any given isolated worm; hence, the observed group effect was not a result of the grouped worms being exposed to less of the irradiated

water. Whatever may be the cause of the longer survival of the grouped planarians, it is evidently not the result of a differential response produced by the amount of irradiated water which was accidentally carried over.

Heilbrunn and Mazia (1936) summarized the somewhat scanty evidence which indicates that treatment with ultra-violet increases permeability. When tissues of many layers are so treated, they recognize that cytolytic changes, rather than reversible membrane effects, may be responsible for the observed changes. The data we have presented, which show that irradiated planarians lose electrolytes into distilled water more rapidly than do nonirradiated controls, do not throw light on this particular point. The electrolytes can be demonstrated to have leached out before lesions were visible on the living worms.

The effects on water produced by worms living or dying and disintegrating give what we call "conditioned water." Tests of the effect of such water on the rate of cytolysis show that, at least as tested, the effect of heavily conditioned water hastened cytolysis, as did more lightly conditioned water if it produced any measurable effect. This is evidence against the observed retardation of cytolysis among grouped worms being a result of the action of something added to the water by the disintegrating worms. Calcium so introduced into worm-conditioned fresh water was shown by Oesting and Allee (1935) to account for the greater longevity of the marine *Procerodes* isolated into hypotonic water. Other evidence at hand concerning the relations of calcium to disintegration of these planarians after treatment with ultra-violet rays can be now summarized and evaluated.

Calcium is known to decrease permeability of cell membranes; hence, if the primary effect of ultra-violet treatment is to increase permeability either by a reversible action on cell membranes or by destructive irreversible processes, a substance which acted to preserve or decrease permeability would probably defer and retard cytolysis.

Irradiated planarians in distilled water disintegrate more rapidly than in the other waters used, all of which contain relatively large proportions of calcium, or of magnesium, which also retards cytolysis in these worms. Cytolysis took place more slowly in well water with 40–50 mg. of calcium per liter than in synthetic pond water of the same pH. The calcium content of such water was approximately half that of well water. The irradiated planarians also survived longer in synthetic pond water containing calcium than in similar water in which magnesium had been substituted and which contained no calcium. When CaCl_2 was placed in well water to make a 0.0083 M solution (disregarding the calcium already present), the planarians survived decidedly longer than in plain well water. This amounts to adding some 334 mg. of calcium to the amount already present and is out of all proportion to the amount added by 10 disintegrating worms in 5 cc. of water.

With the worms in distilled water the protective value of 0.000001 M CaCl_2 could be demonstrated. In this water there was an increase of 0.04 mg. calcium per liter. While we did not find or develop quantitative methods which would accurately determine the amount of calcium present at this dilution, we did get indications that 500 planarians in 500 cc. of water gave off on the order of 0.5 mg. of calcium. If the yield was at the same rate when 10 worms were placed in 5 cc., we could expect that there would be 0.01 mg. of calcium present, which should retard cytolysis, as compared with that shown in water

containing an isolated worm. For distilled water, then, calcium may be one of the factors that caused the observed group-single differential survival.

With well water or the synthetic pond water used, this can hardly be an important agent, since, as was stated earlier, in such waters the conductivity was not measurably changed by the presence of disintegrating worms, originally 1 worm per cubic centimeter. To be sure, a decided increase in the amount of calcium in well water increased survival of the planarians; however, the amount of calcium introduced was out of all proportion to the amount given off by worms that cytolized in distilled water.

The observations recorded earlier show that worms disintegrate more slowly in well water with pH below 7.0 than in more alkaline well water. This water is rich in carbonates. For years we have known that an increase in acidity in water with carbonates may produce effects either directly as a result of the H-ion concentration or as a result of the release of carbon dioxide (Smith and Clowes, 1924). From data given in Table 10 we find that only in carbonate-free well water is pH the apparently effective factor, and here the survival is longer in the lower H-ion concentration. This factor had no effect on survival in synthetic pond water which lacks carbonates. The longer survival in carbonate-containing well water of higher H-ion concentration is probably related to its action in setting free carbon dioxide.

In comparing different types of water of the same or different pH, it becomes clear that total electrolytic concentration, when artificially varied, is an important factor. Length of survival is directly related to the conductivity of the water. It has been demonstrated that animals increase the total electrolytic content when placed in distilled water. In such water, therefore, this could be a mechanism resulting in group protection. Loss of electrolytes into waters of high conductivity has not been shown.

Direct experiments show that well water charged with washed carbon dioxide to pH 6.0 caused worms placed in it to survive the effects of irradiation longer than in well water controls at pH 7.3 or higher. Both these initial pH values drifted. After 3.5 hours, when the worms were approaching total stage 30, the carbon dioxide charged well water was about pH 7.8, while the straight well water showed pH 8.2.

As a result of these observations we have three different mechanisms which may have operated in at least some of the cases in producing the observed differential survival of grouped and isolated animals. In distilled water the larger amount of calcium and other electrolytes leached from the grouped planarians may have been effective in retarding cytolysis. In distilled water and in all other waters used, the more rapid accumulation of carbon dioxide would have the same effect, while in some waters, the higher H-ion concentration about the group may have acted indirectly to lengthen the survival of grouped worms. Much more work would be needed to establish the exact limitations of the role of each of these factors in producing the observed group effect.

The case reported in the earlier sections of this paper, that of group protection for *E. dorocephala* during and after exposure to ultra-violet radiation, adds another to the list of recently demonstrated examples of undercrowding, as summarized by the left-hand limb of the curve in Figure 1, B. This is not the place to discuss the general biological implications of these particular experiments or the phenomena associated with undercrowding as a whole. Certain aspects of this discussion have been undertaken recently by the senior author (Allee, 1938), and we have neither space nor inclination to repeat or summarize this material.

SUMMARY

1. *Euplanaria dorotocephala* can survive exposure to ultra-violet radiation longer, other things being equal, if exposed in some numbers, even though no shading occurs, than if isolated while being irradiated. The worms were always assayed after transfer to fresh water which had not been irradiated.
2. After identical irradiation, groups of 10 planarians survive longer after transfer to a small amount of fresh water than do similar planarians isolated into the same amount of water.
3. As might be expected, planarians irradiated in lots of 10 and transferred together to fresh water survive much better than if kept continuously isolated during and following irradiation.
4. Worm-"conditioned" water, as tested, hastened the cytolysis of irradiated worms.
5. The addition of CaCl_2 delayed cytolysis; in double-distilled water a solution as dilute as 0.000001M was appreciably effective.
6. The group effect was apparent when the grouped and isolated worms were placed in proportional volumes in the usual saltcellar embryological dishes. It disappeared when volumes were proportional and the depth was equal.
7. Agitation of the worms hastens cytolysis and causes the differential survival of grouped and isolated worms to disappear.
8. Planarians tend to cytolize more slowly after irradiation if placed in well water with pH between 6.0 and 6.8 than if in water with pH of 7.3-8.2. This difference is not found in synthetic pond water. The reverse occurs in carbonate-free well water.
9. Irradiation increases the permeability of planaria to contained electrolytes if the worms are placed in hypotonic water; no effects were found in waters with a conductivity of 325×10^{-6} mhos or more.
10. Grouped planarians probably give off enough calcium into distilled water to retard cytolysis significantly; this is probably not a factor with waters already fairly rich in calcium.
11. An increase in carbon dioxide also delays cytolysis after irradiation.
12. We are not prepared to delimit precisely the roles played by calcium, total electrolytes, H-ion concentration, and carbon dioxide in producing the longer survival of grouped planarians.

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REACTIONS OF TADPOLES TO LIGHT. I. EFFECT OF CHEMICALS ON AGGREGATION OF TADPOLES IN UNILATERAL ILLUMINATION

(Eight figures)

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AN ATTEMPT to find out if tadpoles vary in reactivity to light led to the experiments to be described in this paper. In order to carry out this work, it was necessary to obtain a series of measurements that would express quantitatively the degree of sensitivity to illumination and thus permit an exact comparison of their reactivity at different ages. It soon became apparent, however, that there is little or no relation between age and sensitivity. Tadpoles that have just left the mass of jelly that surrounds the eggs are, perhaps, generally less positive than older ones; but, aside from these very young individuals, no evidence of a correlation between age and sensitivity to light was observed.

One thing, however, was evident from the very beginning of the experiments: the sensitivity of tadpoles may differ markedly from time to time, even if not in a progressive manner. A group that is strongly positive today may be much less so or, rarely, may be even negative tomorrow, a behavior which suggests that in their response to illumination these animals are easily affected by the environment. Consequently, they should be excellent subjects for a study of the effects of various reagents on the response to light. It was for this purpose that they were used in the experiments herein recorded.

It has long been known that numerous factors may exert an influence on the response of organisms to light. Of these, perhaps the most important is the intensity of the light itself. Many organisms are positive at one intensity and indifferent or negative at another. Temperature has a marked effect on the reactions of most, and perhaps all, organisms; and, among those that live in the water, the chemical condition of the medium is usually of the utmost importance. But, although these things may have so pronounced an influence, there is a remarkable lack of specificity of effect. One can scarcely make a single generalization that will apply in all cases. Increase in temperature may cause negative animals to become positive; but, on the other hand, it may cause positive animals to become negative. Acids make negative *Spondylomor* positive, and positive ones still more positive (Mast, 1918); but, as we shall see, they make positive tadpoles negative, and negative ones more negative. Numerous examples of such contrasting results could be cited. In spite of the fact that many data have already been collected, it is undoubtedly true that observations on various additional organisms will have to be made before definite and final conclusions can be drawn as to the effect of almost any reagent. It was in the hope of adding something toward the elucidation of the effects of a few reagents that the investigation to be recorded was undertaken. It deals with the action of certain chemicals on the response to light by *Bufo* and *Rana* tadpoles.

METHODS

If one is to relate the reactions of an organism at different times and under diverse conditions, it is obviously advantageous to use some system of quantitative measurement that will admit of accurate comparison. Kanda (1919), in his work on *Arenicola*

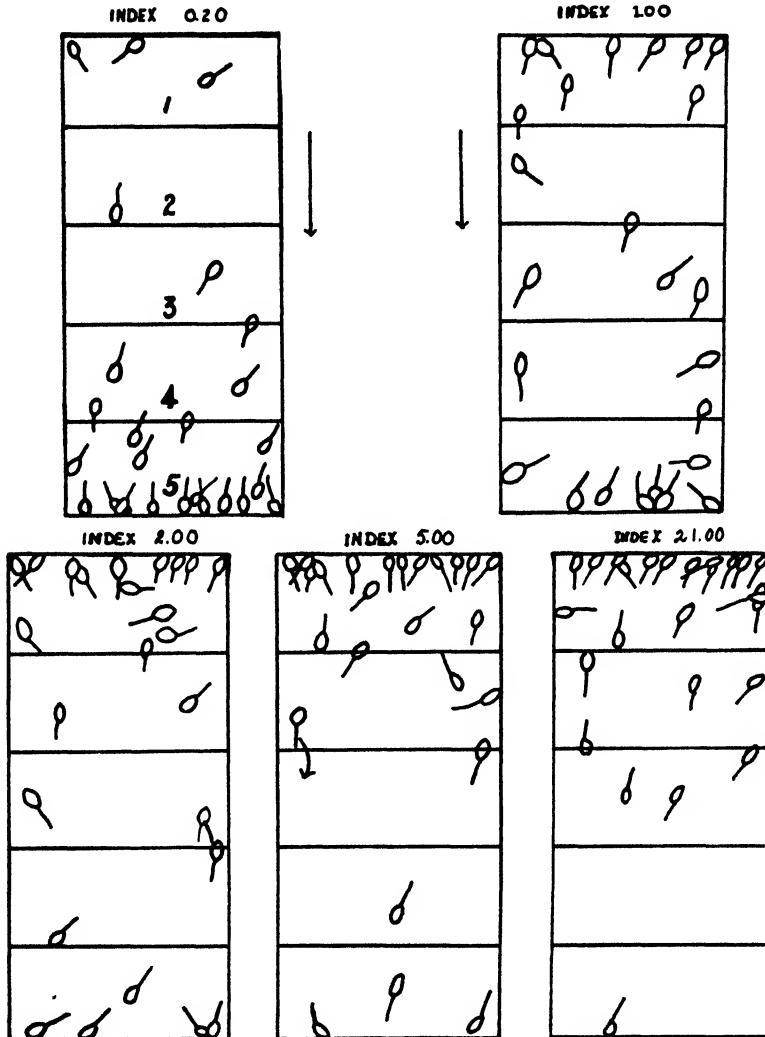


FIG. 1.—Illustrating distribution of tadpoles in dish at various indices of reaction. Direction of light indicated by arrows. (See text.)

larvae, recorded estimates of the percentages of larvae that were attracted to the lighted end of a dish; and these estimates are of more aid to the reader than such expressions as "strongly positive" or "slightly negative." A somewhat more exact method of measurement has been employed in the present instance and will be described below.

Groups of 25 tadpoles were put into 1,000 cc. of water in rectangular museum jars, 12 inches long by $3\frac{1}{2}$ inches wide by 8 inches high, and subjected to illumination, which was furnished by a 200-watt concentrated filament lamp, situated about 8 inches from one end of the jar. Under the latter was placed a piece of black cardboard, $12 \times 3\frac{1}{2}$ inches in size, which was divided into five equal parts by means of white cross-lines. When viewed from above, the white lines stood out conspicuously on the black background and served to divide the jar into compartments. These compartments were numbered from 1 to 5, 1 being nearest the light and 5 farthest from it. The type of reaction could thus be ascertained by comparing the numbers of tadpoles in the various compartments. In some instances, especially when results are expressed in curves, it has been convenient merely to indicate the number of tadpoles at the lighted end of the jar, that is, in compartments 1 and 2. This, however, does not admit a ready comparison of groups of different numbers; and to facilitate such comparison and to condense and simplify results for presentation in tables the following scheme was devised. The number of tadpoles in compartments 1 and 2 were added (*a* of Table 1), and the sum divided by the number in compartments 4 and 5 (*b* of Table 1).¹ The resulting quotient has been called the "index of reaction." It is evident that an index of 1 shows a group as a whole to be indifferent to light, there being then as many animals at one end as at the other. An index of more than 1 indicates a positive group; and of less than 1, a negative group.

In order to help the reader to visualize the relations indicated by the index, a series of diagrams is presented in Figure 1, in which the number of tadpoles in the several compartments is shown, with indices of 0.20, 1.00, 2.00, 5.00, and 21.00, respectively.

Three different species of tadpoles were used in the experiments: toad (*Bufo*), wood frog (*Rana cantabrigensis*), and leopard frog (*Rana pipiens*). There appears to be no specific differences in their behavior, as far as reaction to light is concerned. Anything that affects the responses of one species apparently has the same general effect on the responses of the others. However, because their excessive activity somewhat hindered accurate counting, the tadpoles of the toad were not quite so favorable for study as the others. Those of *R. pipiens* were perhaps the best, but all three of the species could be used and were used to advantage.

EXPERIMENTAL RESULTS

A. PRELIMINARY EXPERIMENTS

Before considering the effects of chemicals on the reaction to light, it may be of some benefit to inquire briefly into the general behavior of tadpoles when they are placed in tap water in a rectangular jar (previously described) which is illuminated from one end.

1. *Variation.*—As one would expect, there is some variation in the reactions of a given group over a period of time, and there may be a very considerable diversity in the reactions of different groups. Examples of such variations are shown in Figure 2, in which are recorded observations on two lots of 25 individuals each. The observations, which were taken every minute, extended over a period of more than half an hour. The

¹ Note that the tadpoles in the middle compartment are ignored, as they indicate neither a positive nor a negative condition. It would have simplified results somewhat if the dish had been merely divided into two parts. The larger number was chosen at the beginning of the experiment, with the prospect that a more detailed record might be of some importance. It was retained because, since a very considerable amount of data was collected with it, it would offer a better comparison of data collected later.

curves shown in the figure denote the numbers of animals at the lighted end of the jar, in compartments 1 and 2. Obviously, there was a difference in the sensitivity of the two groups. One was decidedly positive, as indicated by an average of 20.8 tadpoles at the lighted end. The smallest number at this end at any time during the entire experiment was 16, and the largest number was 24. The second group, on the other hand, was practically indifferent, with an average of 11.6, a minimum of 8, and a maximum of 13 at the lighted end. While the two groups differed every considerably from each other, there was not a very marked deviation in the reactions of either. The average number from almost any five consecutive observations would be very close to the average from

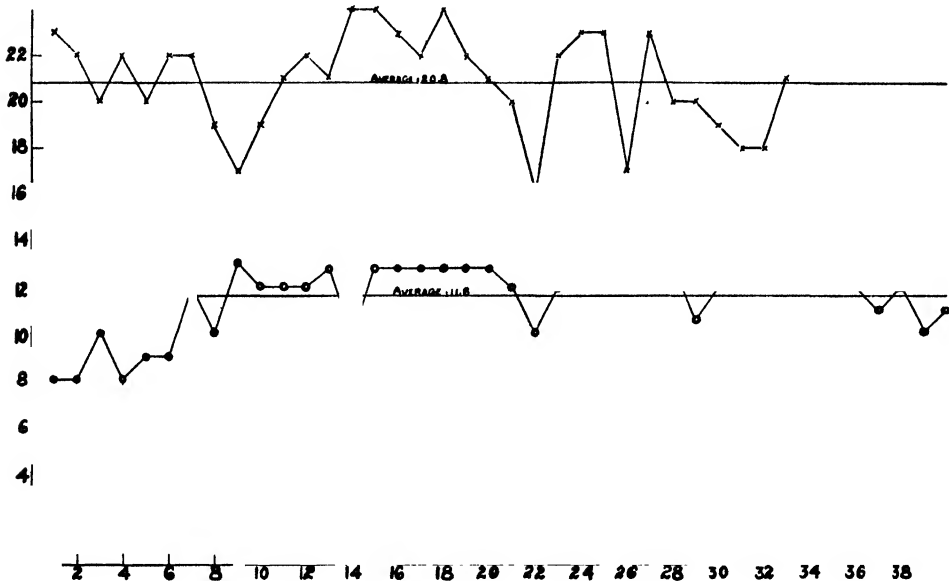


FIG. 2.—Showing variation in numbers at lighted end of dish in two groups of tadpoles over a considerable period of time. Abscissas, time in minutes; ordinates, number of tadpoles (out of a possible 25) at lighted end of dish

all the observations. In experiments to be described, averages have been established from five observations.

As far as variation within individual groups is concerned, the foregoing examples are typical. In very few instances was it found to be materially greater or less. Neither lot, however, could be said to be typical with respect to the quality of reaction, that is, to the degree of positiveness or lack of positiveness with which it responded to light. As a matter of fact, the divergence in the reactions between different groups was so marked that any one group could scarcely be designated as typical. In tap water a few of the many groups experimented with were slightly negative. In general they were positive.

2. *Time required for aggregation.*—From the foregoing results it is evident that tadpoles distribute themselves in a certain way over a dish that is illuminated from one end, and, if undisturbed, tend to retain this distribution. An alteration in the environment, such as a change in the chemical content of the surrounding medium or in the direction

of the light rays may lead to a redistribution. In Figure 3 are shown the results of an experiment designed to ascertain the time required for reaggregation after the direction of the light was reversed. In this experiment two 200-watt lamps were placed at opposite ends of a jar. Alternately one was turned on for a few minutes and the other turned off. Twenty-five tadpoles were put in the jar, and observations were made every minute. The numbers recorded are those of animals in the two compartments at the left end of the dish. As indicated in the figure, the left light was first turned on, and three observations made. The first observation showed 17 animals at the left end; the second, 19; and

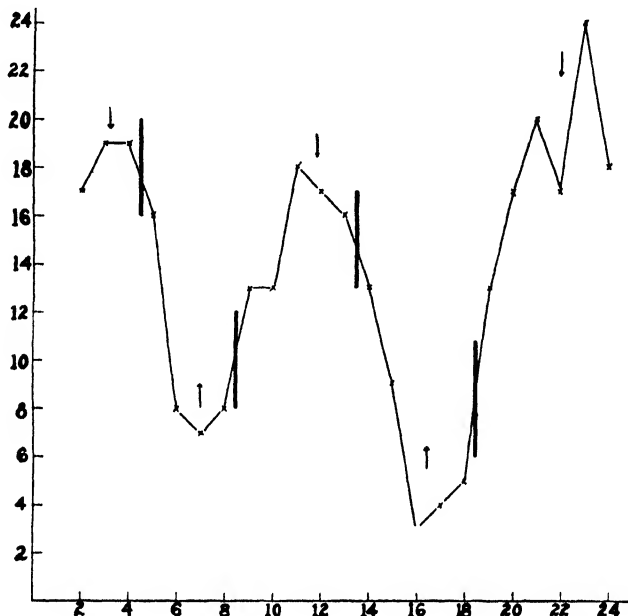


FIG. 3.—Showing time for reaggregation when direction of light is reversed. Lamps placed at both ends of dish, and first one turned on, then the other. Heavy vertical lines indicate time at which light was changed. Abscissas, time in minutes; ordinates, number of tadpoles at left end of dish. Arrows indicate direction of light.

the third, 19. The left light was then turned off, and at the same time the right one was turned on. Four observations showed 16, 8, 7, and 8 animals, respectively, at the left end. Again the lights were reversed; and five observations found 13, 13, 18, 17, and 16 animals at the left end, which was now again the illuminated end. As the figure indicates, two more reversals of the light gave the same results, namely, a redistribution of the tadpoles within 1 or 2 minutes after the position of the light was changed.

B. EFFECT OF CHEMICALS

1. *Eosin*.—Of the various reagents whose reactions were investigated, eosin proved to be one of the most efficient in influencing the reaction to light. Its effects are clearly shown in Table 1. Since the results set forth in this table also illustrate the general

method of procedure in several subsequent experiments, it will be well to examine them somewhat in detail. As indicated, six different lots of tadpoles were used, one of them twice. In the case of each lot, five, and in one or two instances four, observations were made at intervals of about 15 minutes, and the number of animals in the several compartments was ascertained. Enough eosin was then added to give a decided tinge to the water; and, after 30 minutes, five more readings were made, with the same time-intervals as before. The jar was reversed after each reading. The figures in the table are the averages of these various readings.

Group 25 might be selected as an example fairly illustrating the effect of eosin. Before treatment there was in this group an average of 15.5 tadpoles in compartments 1 and 2,

TABLE 1

SHOWING THE EFFECT OF EOSIN ON THE REACTION TO LIGHT BY TADPOLES

Each group was composed of 25 animals (21 in the case of No. 101). Treatment consisted in adding sufficient eosin to give a marked color to the water containing the tadpoles. Details of procedure are given in the section "Methods."

NO. OF GROUP	NOS. OF ANIMALS IN VARIOUS COMPARTMENTS										INDEX OF REACTION (a/b)	
	Before Treatment					After Treatment						
	a		3	b		a		3	b		Before Treat- ment	After Treat- ment
	1	2		4	5	1	2		4	5		
101 (wood frog).....	9.8	2 6	2.0	2.4	4.2	0.8	0.8	1.2	3.0	15.2	1.88	0.09
25 (wood frog)....	14 25	1.25	2.5	2 0	5.0	3.75	0 25	0.0	1.25	19.75	2.21	0.19
111 (wood frog)....	14.8	1.8	1.2	1.6	5.6	1.2	0.6	2.0	2.6	18.6	2.31	0.08
111 (wood frog)....	6 8	2 6	2.6	4.8	8.2	0.25	0.25	0.75	5.75	18.0	0.72	0.02
104 (toad)	16.8	1.8	2 6	1.6	2.2	10.4	2.6	3.2	1.6	7.2	4.89	1.48
109 (toad)	10 0	3.4	1.6	1.6	8.4	6.4	2.8	1.8	3.6	10.4	1.34	0.66
107 (<i>Rana pipiens</i>)..	6 2	2.8	2.8	3.4	9.8	1.2	0.8	3.4	5.2	14.4	0.68	0.10
Average.....	11.2	2.3	2 2	2.5	6.2	3.4	1.2	1.8	3.3	14.8	1.55	0.25

and an average of 7 in compartments 4 and 5, giving an index of 2.21. Introduction of the eosin brought about a conspicuous change in reaction. In compartments 1 and 2 the average number of tadpoles fell to 4, and in compartments 4 and 5, rose to 21, reducing the index of reaction to 0.19. Thus the dye had the effect of causing a decidedly positive group to become strongly negative. Examination of Table 1 will show a similar effect in every instance. Group No. 104 was very positive, with an index of 4.88. To be sure, eosin did not cause it to become negative, but it did greatly lower the positive reaction, decreasing the index to 1.48. Two groups, Nos. 111 and 107, were negative before treatment. In each instance eosin caused a further decrease in the negative reaction, reducing No. 111 from 0.72 to 0.02 and No. 107 from 0.68 to 0.10. All the other groups were changed from positive to negative. The average of all the groups before treatment was 1.55; after treatment, 0.25. Clearly, eosin tends to make positive tadpoles negative, and negative ones still more negative.

It has already been stated that untreated groups of tadpoles, that is, groups which have been in tap water that is slightly alkaline, are generally positive. Of the six groups we have just noted, four were decidedly positive before treatment, one was negative, and the other, No. 111, the only one that was used twice, was first positive and then negative. As indicated, No. 111 was first distinctly positive. Treatment with eosin made it negative, as one would expect. Left in the eosin overnight, it was removed to tap water 2 hours before its reaction to light was again tested; but 2 hours was not long enough for complete recovery from the effects of the dye. Given sufficient time in the tap water, it undoubtedly would have become positive again. Thus, only one group, No. 107, was

TABLE 2

SHOWING THE EFFECTS OF VARIOUS CHEMICALS ON THE REACTION
TO LIGHT BY TADPOLES

Each experiment included ten observations, five before and five after the introduction of the chemical. For method of obtaining index of reaction, see Table 1.

CHEMICAL USED	NUMBER OF EXPERIMENTS	INDEX OF REACTION		$\frac{x}{y}$
		Control (x)	Treated (y)	
Ammonium hydroxide	7	1 10	4 10	0 27
Sodium hydroxide	12	2 01	2 21	0 91
Urea	10	2 18	2 02	1 08
Ether	9	4 17	3 31	1 26
Sucrose	14	2 74	2 11	1 30
Sodium chloride	4	2 01	1 31	1 53
Dextrose	13	2 50	1 62	1 54
Ethyl alcohol ($\frac{1}{2}$ of 1 per cent)	14	2 48	1 43	1 73
Sulphuric acid	14	3 69	1 65	2 24
Methyl alcohol (1 per cent)	22	2 55	1 11	2 30
N-propyl alcohol ($\frac{1}{2}$ of 1 per cent)	9	3 04	1 45	2 51
Eosin	7	1 55	0 25	6 20

really negative before treatment. Experience has shown that the proportion of negative untreated groups is even much lower than indicated by these numbers.

It so happens that group No. 107 was composed of tadpoles of *R. pipiens*, the only tadpoles of this species used in the experiment. That it was negative was apparently merely fortuitous, as further observations have given no reason for believing that the tadpoles of this species are more likely to be negative than those of *R. cantabrigensis* or *Bufo*.

2. *Comparison of various chemicals.*—That eosin is not the only substance that appreciably affects the reaction of tadpoles to light is clearly apparent from Table 2, which shows the effects of the several chemicals that were investigated. The procedure in these tests was precisely like that in the experiment just described; that is, the reaction of the untreated group was first found, the chemical then added, and after half an hour the reaction again ascertained. As may be seen, ammonium hydroxide was the only substance used which caused a notable increase in the positive reaction. Seven lots of tadpoles were employed to ascertain the effect of this reagent. Before being subjected to ammonium hydroxide, several of them were made negative with eosin or acid; and as a consequence

the average index of reaction of the seven groups was low. The action of the ammonium was very pronounced. Every positive group was made much more positive, and every negative group was made positive. The general average was raised from 1.10 to 4.10.

Sodium hydroxide does not appear to be nearly so potent as ammonia in influencing the response to light. From the data presented in Table 2 there might even be some question as to whether it has any effect at all. The average reaction of the ten groups tested was 2.01 before treatment and 2.21 after treatment, a difference which is perhaps within the limits of experimental error. As a matter of fact, these figures are misleading. It will be demonstrated later that sodium hydroxide actually does have a marked influence on the reaction to light.

With the possible exception of urea, all the other chemicals that were tested tend to make positive groups less positive or negative, though with varying degrees of efficiency. And even urea lowered the index of the reaction from 2.18 to 2.02, a difference which is not great enough, however, to be of much significance. Ether lowered the index of the reaction from 4.17 to 3.31; sucrose from 2.74 to 2.11; sodium chloride from 2.01 to 1.31; and dextrose from 2.50 to 1.62. Various concentrations of these chemicals were employed, and the foregoing figures might be materially changed by picking out and using the concentration that is most effective. Three alcohols—methyl, ethyl, and propyl—were used. All three proved to be fairly efficient in reducing the index of the reaction, 1 per cent methyl changing it from 2.55 to 1.11, three-fourths of 1 per cent ethyl from 2.48 to 1.43; and three-eighths of 1 per cent propyl from 3.64 to 1.45. Sulphuric acid also lowered the index of the reaction rather markedly, namely, from 3.69 to 1.65. We have already noted the effect of the eosin.

To facilitate somewhat a comparison of the relative effects of the different substances that were used, the indices of the reaction obtained before treatment has been divided by the indices obtained after treatment, and the quotients placed in the final column of Table 2. It should be kept clearly in mind, however, that this comparison means little except in a very general way. Undoubtedly eosin, generally speaking, is more effective than sodium chloride or dextrose in causing tadpoles to become less positive to light. But, while 1 per cent methyl alcohol is evidently more efficient than three-fourths of 1 per cent ethyl, methyl can hardly be ranked above ethyl for that reason, as other concentrations might very likely have reversed their relative effectiveness.

3. *Sulphuric acid.*—Although in a large number of instances several of the foregoing chemicals, especially those at the bottom of the list, such as the alcohols or sulphuric acid, made positive groups negative, it is evident from Table 2 that, with the exception of eosin, they generally did not: they usually made slightly positive groups negative, and strongly positive groups less positive but not negative. Eosin thus appears to be more potent in effecting a change in reaction than any other substance that was used.

"Appears" has been used advisedly in the preceding sentence, for further experience has shown that the most noteworthy effects of sulphuric acid, and ammonium and sodium hydroxides as well, are to be observed within a few minutes after the chemical has been introduced, and so cannot be discovered with a procedure such as the one described, in which no observations were made until 30 minutes after the chemical was added. This early effect of sulphuric acid is clearly illustrated by the results of an experiment which are presented in Figure 4. In this experiment 69 observations were made at intervals of 1 minute—23 of them before the acid was added, and the remainder after. A group of 25 tadpoles was used as usual. Time is represented in the figures by the

abscissas; and the number of animals at the lighted end of the jar, in compartments 1 and 2, by the ordinates. As may be seen, the group was decidedly positive to begin with, there being from 17 to 19 animals at the lighted end of the vessel. It remained equally positive for 3 minutes after the introduction of the acid, then on the fourth minute the number at the lighted end suddenly fell to 4. The group had thus become strongly negative, and it continued in this condition for 6 minutes. Then it gradually became less negative, until on the seventeenth minute after the addition of the acid the number in the first two compartments had reached 14. The group was positive again, but only

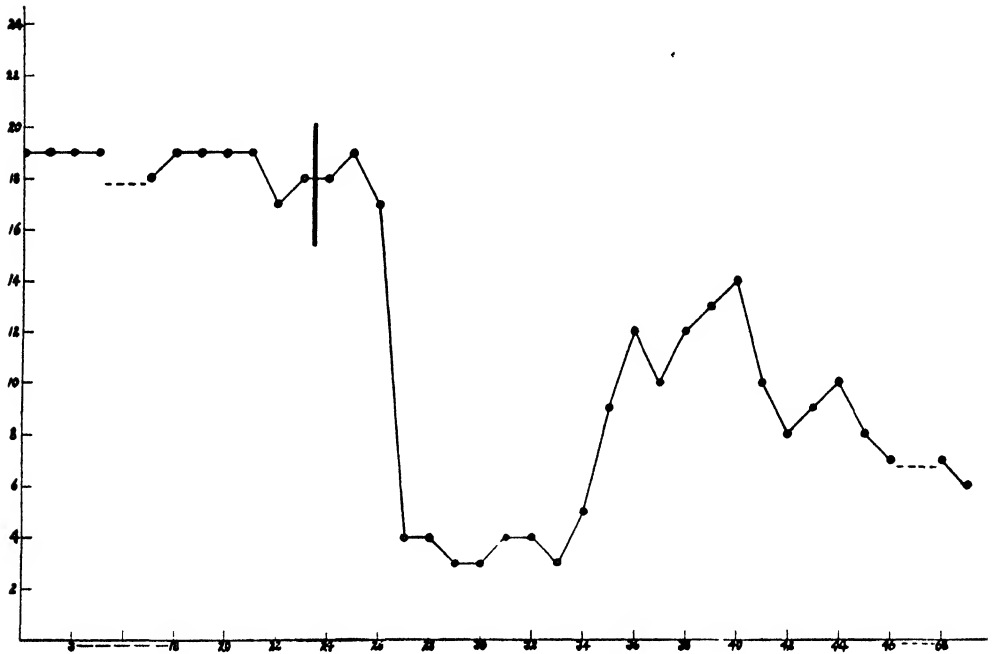


FIG. 4.—Effect of sulphuric acid on reaction to light. Abscissas, time in minutes; ordinates, number of tadpoles at lighted end of dish. Heavy vertical line indicates time at which acid was added to water containing the tadpoles.

temporarily, for the number of tadpoles at the lighted end began to decrease, until in a few minutes there were but 7 left; and this negative condition persisted for the remainder of the experiment.

The effect of acid on the reaction to light thus appears to be clear-cut. To show that the peculiarities just described are characteristic and not merely accidental, the reactions of four additional groups are indicated in Figure 5. All the curves in this figure were constructed as was the curve in Figure 4, and all denote the same type of behavior as described above. In addition, still other experiments were made, and almost invariably with the same general result. Within 1-4 minutes after introduction of acid there was a rapid lessening in the number of animals at the lighted end of the jar, sometimes, as shown in curves 2 and 3 of Figure 5, there being none at all left at this end. Then followed the curious postreaction, and the tadpoles surged back to the lighted end until

there were almost as many, or, in a few instances, fully as many, as before the addition of the acid. However, in a very short time (usually within 1 or 2 minutes) they began to retreat, with the final result, as noted above, that the group became either negative or much less positive than it was before treatment.

4. *Ammonium and sodium hydroxides.*—It has already been noted that ammonium hydroxide brings about a decided increase in the positive reaction. Ammonium hydroxide is like the acid, however, in that its most pronounced effects are to be observed shortly after its introduction into the water. This may be clearly seen from the experiments on two groups of animals, the results of which are presented in Figure 6. The procedure in these experiments was similar to that in the experiments with sulphuric acid just

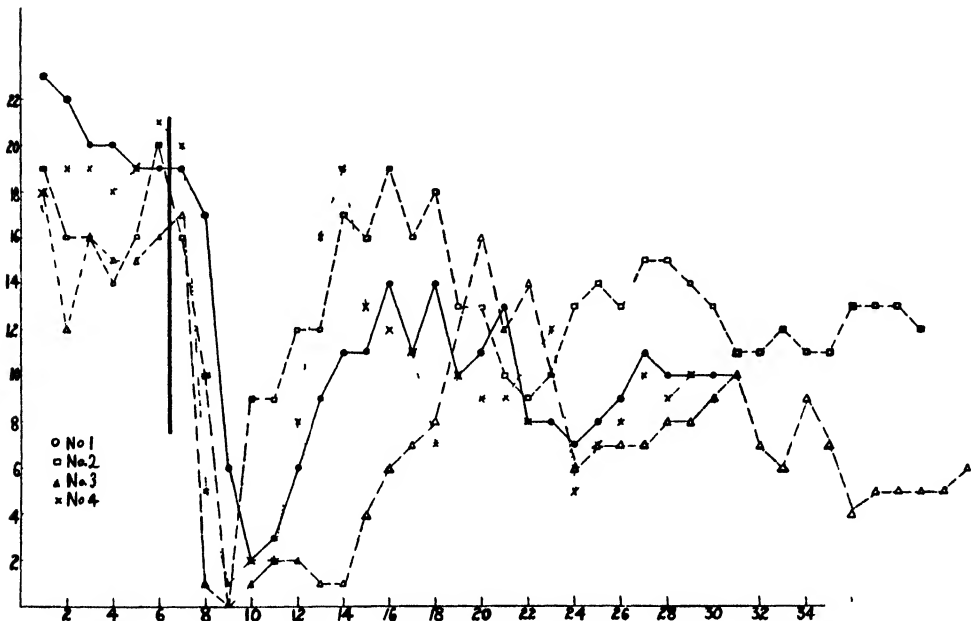


FIG. 5 — Effect of sulphuric acid on reaction to light See Fig. 4

described. Groups of tadpoles were placed in tap water in the museum jar, a number of readings were taken at intervals of 1 minute, a small amount of ammonia was added to the water, and the observations were continued for 20 or 30 minutes longer.

Curve 1 of Figure 6 well illustrates the effects of ammonium hydroxide. As may be seen, the group used in this experiment was at first slightly positive. Before introduction of the chemical there were from 13 to 17 animals at the illuminated end of the dish. Ammonium hydroxide had the immediate effect of making the group very strongly positive, the second observation after the addition of the chemical showing 23 animals at the lighted end. Gradually, however, it became less positive, until 4 minutes later it was distinctly negative, with only 6 larvae at the lighted end. This condition also proved to be temporary, and soon the group was positive again. Finally, 20 minutes or so after the addition of ammonia, the number at the illuminated end was in the neighborhood of 20. The group was not only positive but considerably more so than before treatment, and it

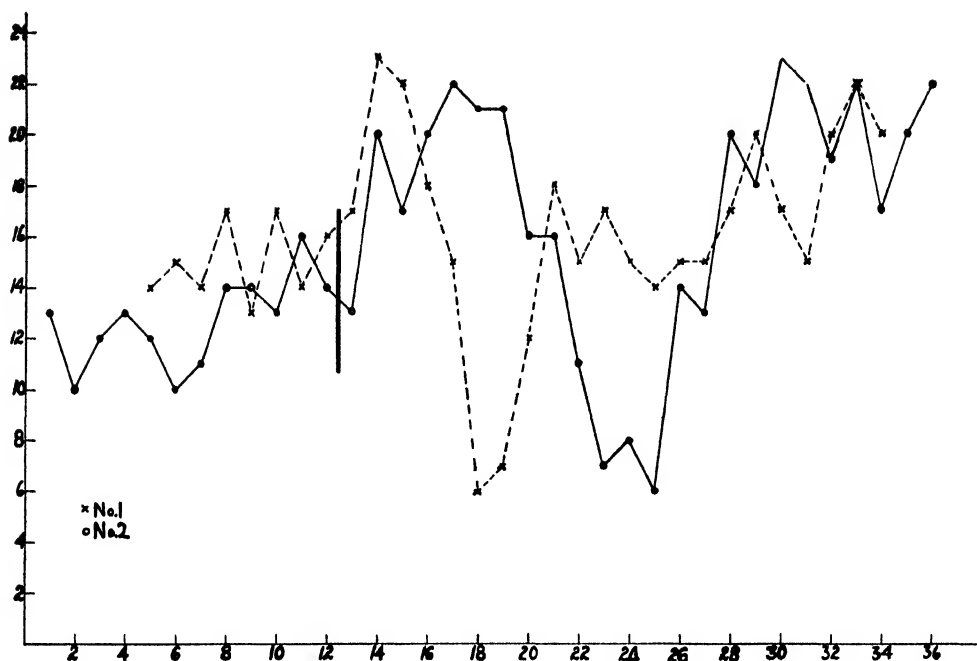


FIG. 6.—Effect of ammonium hydroxide on reaction to light. Constructed as in Fig. 4

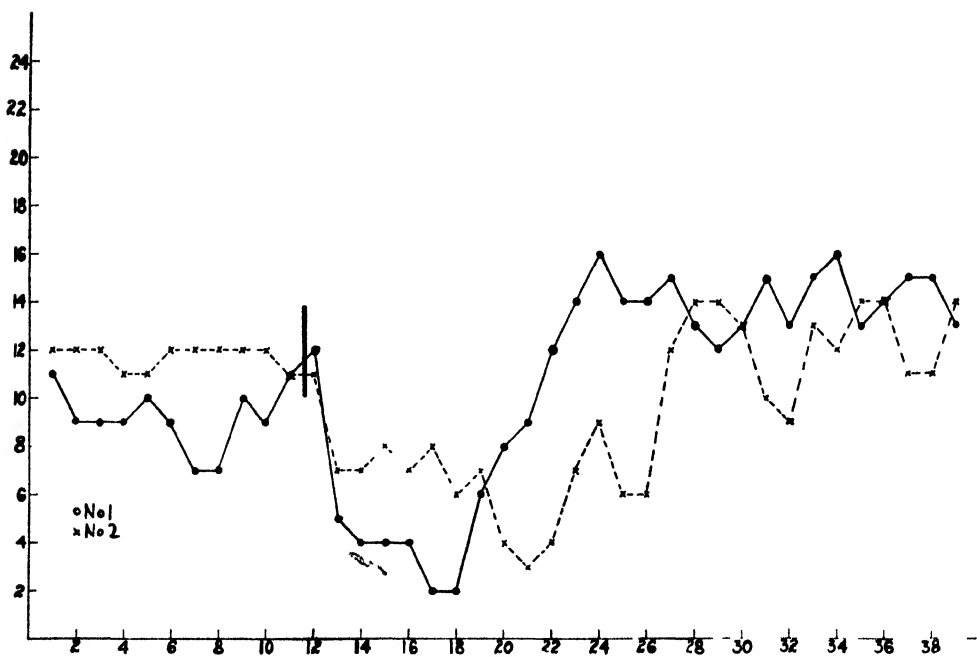


FIG. 7.—Effect of sodium hydroxide on reaction to light. Constructed as in Fig. 4

remained in this condition. Curve 2 illustrates the same general behavior in another group.

Sulphuric acid and ammonium hydroxide thus appear to be opposed in all their effects. The acid causes a marked decrease in positive reaction, followed by an increase, followed, in turn, by a decrease. The alkali causes an increase in positive reaction, followed by a decrease, followed by an increase. The final effect of the acid is to make a group less positive; of the alkali, is to make it more positive.

As may be seen from Figure 7, the effect of sodium hydroxide differs qualitatively, as

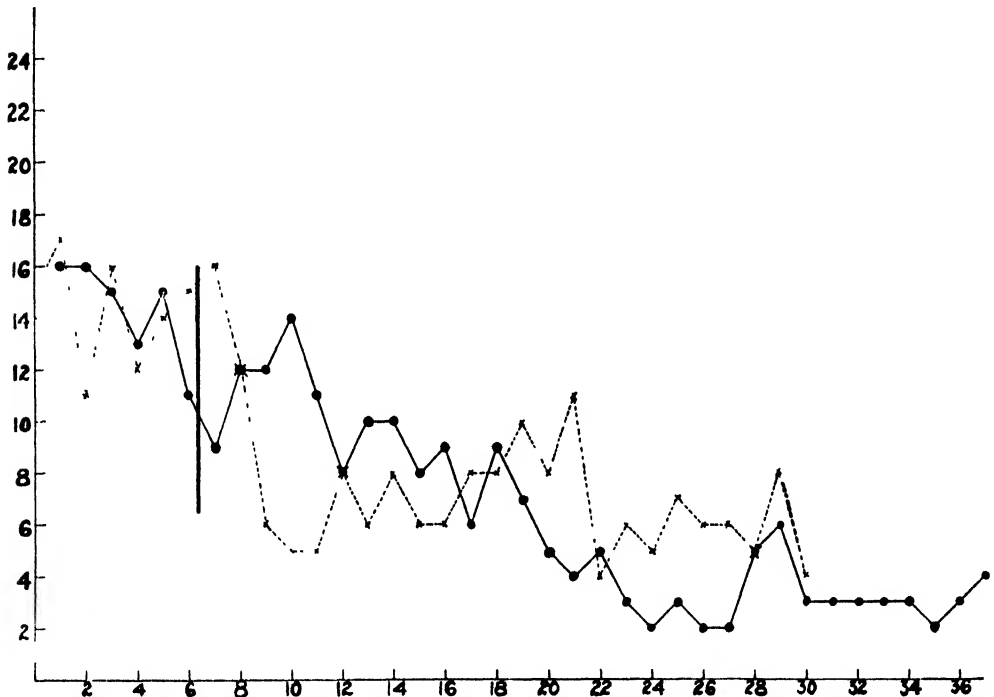


FIG. 8.—Effect of eosin on reaction to light. Constructed as in Fig 4

well as quantitatively, from that of ammonium hydroxide. The group used in the experiment which is illustrated in curve 1 was, as shown, slightly negative before treatment, with an average of 9 or 10 animals at the illuminated end. Sodium hydroxide had the immediate and decided effect of causing it to become much more negative; within 2 minutes after its introduction the number at the lighted end had dropped to 4, and 3 minutes later to 2. This reduction was followed by an increase, and 13 minutes after the addition of the alkali the number at the lighted end was 14. The group was now slightly positive, and it continued so for the remainder of the experiment.

Curve 2 indicates the same type of behavior. Introduction of the sodium hydroxide brought about a reduction in the number of tadpoles at the lighted end from 11 or 12 to 3, but within 16 minutes the number was back to 12. There was no further change for 24 minutes, when the experiment was discontinued.

From these results, as well as from those previously presented in Table 2, it is apparent that the final effect of sodium hydroxide is not very marked. Half an hour after its introduction the tadpoles exhibit about the same sensitivity to light as before treatment, or, at most, are only slightly more positive. In immediate effect, however, this alkali is almost or quite as effective as sulphuric acid and, like the acid, at first induces the tadpoles to become strongly negative. It differs from the acid in that the strong negative reaction is followed by a return to positive reaction that is permanent.

In view of the very peculiar behavior induced in tadpoles by an acid or alkali, it would seem of interest to investigate in detail the actions of other chemicals, especially of eosin, which, as we have seen, has so pronounced a final effect. The results of two experiments in which observations were made at minute intervals, and in which the tadpoles were treated with eosin, are shown in Figure 8. It is clearly evident from this figure that no devious changes in sensitivity, such as sulphuric acid or ammonia bring about, followed the introduction of the eosin. The reactions of the animals were changed conspicuously, to be sure, but in a direct manner. In both instances the number of animals at the illuminated end of the dish was about 15 before treatment, and in both instances the addition of the eosin resulted in a straightforward, gradual decrease in this number to about 5. The one group required only 3 minutes to bring about this change; the other required 10 minutes or so. As the amount of eosin that was added was not precisely controlled, difference in concentration may account for the variation in time.

If a group of tadpoles is made negative by eosin, they remain negative for at least 24 hours, and they will probably remain so indefinitely if retained in the eosin solution.

The effect of the alcohols appears to be similar to that of eosin, although not nearly so marked. Alcohols simply cause a group to become less and less positive, until finally a minimum is reached. The minimum doubtless depends on the concentration of the alcohol.

DISCUSSION

We have been speaking of groups of tadpoles as being positive or negative but have not explained just what that means with respect to the individuals concerned. According to the scheme adopted, an index of 3 indicates that at any given time there are about three times as many tadpoles at the lighted end of the dish as at the opposite end; but it by no means indicates that three-fourths of the animals are positive and remain indefinitely near the light while one-fourth are negative and stay away from the light. As a matter of fact, there is a continuous interchange of individuals from one end of the dish to the other. A given individual is likely to spend part of the time at one end and part of the time at the other. But if the group to which it belongs is positive, it will probably be near the light most of the time; while, if the group is negative, it is likely to be at the unlighted end of the dish most of the time.

The fact that the reactions to light by tadpoles are influenced by the chemical condition of the medium is not only not surprising but, in view of the findings of numerous other investigators, to be expected. The peculiar behavior induced by acids and alkalis, however, appears to be unique, although Mast (1918) obtained results somewhat similar in the reactions of *Spondylomorum*. He found that these organisms, which were negative at a given intensity of light, could be made positive by treatment with acid, a condition which proved to be temporary, for soon they were negative again. Alkalies had no effect on the reaction.

It is apparent that the experiments with acids and alkalies discussed in this paper are not complete. In every case the experiments were begun with the animals in tap water, which was slightly alkaline. Under this condition, as noted, the final effect of sodium hydroxide was not very noticeable. But it is possible, as there is some evidence for believing, that if, instead of being in alkaline tap water, the tadpoles had been in an acid solution and thus rendered less positive, the final effect of the sodium hydroxide would have been much more pronounced. Any final conclusion, however, awaits further experimentation.

Several theories have been offered to explain the changes in reaction to light that are brought about by chemicals. We have no evidence to show that any of them do or do not apply to tadpoles, except, perhaps, in the case of acids and alkalies. Here the reaction appears to be too complex to permit a simple explanation, such as, for instance, an increase or decrease in permeability. However, the discovery of Allee and Stein (1918), who found that reversal of light reactions in May-fly nymphs was accompanied by change in rate of metabolism, might apply—at least in part. It is certain that the activity of the tadpoles is greatly stimulated, both by sulphuric acid and by the two alkalies that were used; and this might account for the initial reversal. Ordinary observation does not show any further change in activity for some time, although there are changes in reaction to light. However, refined measurements might disclose alterations in metabolic rate during this time.

SUMMARY

1. When a group of tadpoles is placed in tap water in a rectangular aquarium jar, at one end of which a 200-watt lamp is located, the tadpoles usually tend to aggregate at the lighted end of the jar. The group can be said to be positive. Occasionally it is indifferent or even negative.

2. Although the degree of positivity, as indicated by the number of animals at the lighted end of the jar, may vary greatly in different groups, and even in the same group from day to day, it remains constant over a considerable period if the environment remains constant.

3. Various chemicals affect the aggregation of tadpoles in light. If ammonium hydroxide is added to the water and observations made half an hour later, the tadpoles are more positive than they were before the addition. Under the same condition, sodium hydroxide seems to have little effect. Urea probably makes tadpoles slightly less positive. Ether, sucrose, sodium chloride, dextrose, sulphuric acid, and methyl, ethyl, and propyl alcohols make positive groups decidedly less positive.

4. Detailed observations show that sulphuric acid causes tadpoles to become strongly negative a few minutes after its introduction. Soon they become positive again, sometimes as positive as before treatment. Then they become less positive or even negative.

5. Sodium hydroxide causes tadpoles to become strongly negative within a few minutes after its introduction. Later they become positive again, so that the final effect of this alkali is not very marked, even though its immediate effect is very much so.

6. The effects of ammonium hydroxide are opposite to those of sulphuric acid. Upon its introduction the tadpoles become more positive, then within a few minutes much less positive or even negative, after which they become strongly positive again, more so than at the beginning of the experiment.

7. Eosin and the chemicals that were used other than the alkalies and acid appear to have no such involved effects as these. Although they may cause positive groups to become less so, their action is direct.

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SOME FACTORS WHICH AFFECT THE RATE OF MOVEMENT OF THE WINGS IN *DROSOPHILA*¹

(Three figures)

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THE purpose of this paper is to point out certain influences which may alter the wing-rate of an insect and to attempt some evaluation of those which lend themselves to analysis. A thorough knowledge of the sources of variation in wing-rate would enable us to judge not only the nature of insect flight but also the organization of the entire animal much more correctly than is possible at present.

The existence of variation in the wing-rate of a species or of an individual insect was recognized by the earliest workers, Landois (1867) and Marey (1868), and by practically every student who has since dealt experimentally with insect flight. At the same time, there has been a tendency among those who have written of insect wing motion to assume that there exists a definite rate which is natural to each species, and that any variation represents a departure from the normal. Occasionally this assumption is made the basis for false theories of insect flight and insect physiology. The publication of rate measurements, accompanied by only slight indications of the conditions under which they were obtained, has contributed to this situation. It is therefore desirable to show, beyond doubt, that variation is normal, to define the normal limits of variation in one species, and to make a preliminary investigation of some of the underlying causes.

MATERIAL AND METHODS

Specimens of *Drosophila repleta* Wollaston were selected at random from a stock which has maintained itself in one of the animal rooms throughout the year. It is felt that it is of particular interest to give in this first survey some idea of the variation in rate to be found in a more or less natural community, and that efforts to control the factors of heredity and metabolic age may wait until such an exploration has been made. Sex was recorded in each case, although the observations show no correlation with this factor. Size was not measured because it was observed that size is no criterion of absolute wing-rate under the conditions given. The experiments were run during the months of February and March, and no seasonal variation became evident. Altogether, several hundred individuals were used in the experiments.

Wing-rate was measured with an electric stroboscope. The method, which is discussed elsewhere (Chadwick, 1939), consists in tuning an intermittent source of light to synchrony with the wing beat. When flash-frequency and wing-rate are synchronous, the wings appear to stand still, and the rate may be read directly from the dial of the instrument. In order to make such observations of wing motion the specimen must usually be

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placed close to the source of light. It may either be "fixed," i.e., held fast, yet free to move the wings, or it may be confined within a small space. For detailed study of thoracic movements, which are of very small magnitude, only the first of these alternatives is applicable. Rate measurements, however, may also be made, though with more difficulty, on insects made to fly in glass jars or other containers. Some measurements have been possible on larger forms, such as Tipulidae, flying about the room. The rate observations recorded below were made on fixed specimens except where there is a definite statement to the contrary.

Observation of fixed and free specimens of *Drosophila* and other insects satisfy the writer that the wing motion is essentially similar, but not identical, under both conditions. One must always take into account that (1) a fixed insect is an insect with a full load and (2) a fixed insect is nearly always "bothered" by whatever holds him fast.

In many of the experiments it was deemed unavoidable to have the specimens fixed. It was essential that the thorax and legs be left free, first that the motions of these parts might be unhampered and visible, and then because contact of the tarsi with an object usually inhibits flight (Fraenkel, 1932). Small stiff paper points, such as are used for mounting beetles, were tipped with a minute amount of paraffin. The specimen was immobilized with an exposure of $1\frac{1}{2}$ –2 minutes to 0° C., tests having indicated that this has no harmful effect; and the paraffined tip was applied under binoculars to the posterior dorsal region of the abdomen and just touched with a hot needle. This doubtless killed the epidermal cells immediately beneath the paraffin (Wigglesworth, 1937); but this result may have been of advantage, in the event that tactile receptors were thereby incapacitated. Care was taken to perform the operation in a similar way on all specimens. Animals whose flight responses were evidently influenced by the nature of the mount were discarded.

For observations on free flight, unmounted specimens were confined in a trough 3 feet by 6 inches by 6 inches, which was illuminated from the ends. Here they showed a positive phototaxis but were not stimulated to fly unless the light intensity was above a definite, though unmeasured, level. The Strobotac with neon bulb was used at one end of the trough. At the other, an ordinary 100-watt bulb, its brightness cut down by ground-glass filters, supplied whatever additional light was needed to raise the total intensity to flight level. The sides and bottom of the trough were lined with red paper, which reduced glaring high lights toward which the insects seemed attracted. A white stripe $\frac{1}{2}$ inch in width was left down the middle of the bottom and appeared to help in keeping the specimens on a relatively straight course. Top and ends of the trough were covered with Cellophane. With this arrangement enough rate readings were obtained to check the results with fixed specimens given below.

It is evident that the general behavior of insects, and hence presumably their wing-rate, is influenced to a large extent by sensory stimuli. For this reason the rate observations on fixed specimens were made regularly with no illumination other than the Strobotron. The intensity of this light varies slightly with the frequency, an unavoidable difficulty; but such variation had no observable effect on the wing motion. Check experiments with varied intensities of illumination were likewise negative. Unnecessary movements were avoided.

The apparatus emits a constant hum of the frequency to which it is tuned, but no response to this or other noises was observed at any time in this species.

Olfactory stimuli were left out of account, it being assumed that the experimental

conditions were reasonably constant in this respect. An attempt to orient freely flying *D. repleta* with the odor of amyl acetate failed.

The existence of a static sense has been looked for regularly but has not been attested in any of the experiments run to date. It is planned to make this question the subject of another communication. The halteres, often mentioned in this connection, must also receive separate treatment. It may be stated definitely that static responses have played no part in the results recorded below.

The responses of *D. repleta* to tactile stimulation during fixed flight are varied. Flight ceases regularly when contact is made with the tarsi, but the tibiae and femora may often be touched with a needle without evoking any response. Touching regions of the abdomen or thorax, or interfering with the wings, may lead to an irregular increase in the wing-rate, followed on continued stimulation by the inhibition of flight and the onset of other responses evidently directed toward evasion. If the head is touched or objects brought near it, this results usually in movements of the forelegs. Flight is inhibited if they succeed in grasping the object. Air currents are also to be classed in the category of tactile stimuli (Fraenkel, 1932; von Buddenbrock and Friedrich, 1932; Hoffmann, 1936) and, since they stimulate flight, may be useful to the investigator. In the present series of experiments, however, all tactile stimuli were avoided in so far as possible. Drafts in the vicinity of the apparatus were shut off by screening. The specimens were not inclosed under glass; this could have been done and would have eliminated chance air currents resulting from movements of the observer, but it also would have made manipulations less convenient and would not have prevented air currents due to movements of the specimens themselves. Flight was induced by picking the mount up suddenly with a pair of forceps, and the specimen was held steady while rate readings were being made. An alternative to this procedure, which has been found useful in photographing flight, has been to fix the mount firmly in such a way that the tarsi of the specimen make contact with the armature of an electromagnet. Closing the circuit depresses the bar, frees the tarsi, and usually results in flight.

After each observation the specimen was returned to the ground on an even keel and released headfirst into an empty vial. Here it remained until needed again.

The observed effects of ordinary sensory stimuli may be summarized by saying that their influence on wing-rate is transient and of small magnitude. Fluctuations from such sources may amount to ± 10 per cent of the basic level (see Table 2, *D*). We presume these fluctuations to be the composite result of the various excitatory and inhibitory stimuli acting on the organism at a given moment, but in practice it is not often possible to analyze the contributory factors separately. The motion of the wings is itself a dynamic phenomenon, entailing tactile and proprioceptive stimulation in the moving parts and metabolic changes in the organism. These considerations are an essential preliminary to any attempt to study the effects of other influences on the wing rate.

THE EFFECT OF FATIGUE

Specimens of *D. repleta*, mounted as described above, were stimulated to fly by means of the tarsal reflex, and the mount fixed in position beneath the binoculars. Flight continued until it ceased spontaneously. Lighting was with the Strobotron only. Rate observations were made at 10-second intervals. Room temperature was constant within ± 0.1 C. Representative results are plotted in Figure 1.

The longest record obtained was 39 minutes. Records of over 30 minutes are not uncommon. Flights of this duration are hardly to be thought of as part of the natural behavior of the species, and it is therefore rather surprising to find such individuals capable of something in the neighborhood of 486,000 consecutive wing strokes (calculated from Fig. 1, No. 2), especially under fully loaded conditions. In these graphs the more superficial type of rate variation shows to advantage, though it has been smoothed out somewhat by the lapse of 10 seconds between successive readings. The differences and similarities between random individuals are also brought out well. The general downward trend of the curves is inferred to be due to fatigue. At the close of such experiments the specimens could be induced to fly again, but only at low rates and for a few seconds at a time. Generally they survived these flights by only a few hours, while

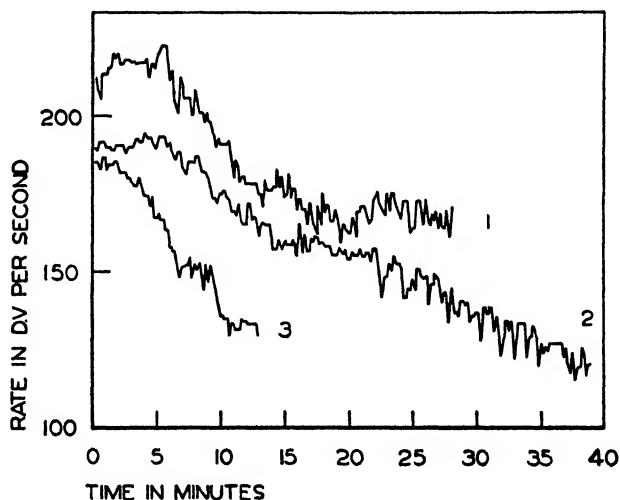


FIG. 1.—Variation in wing-rate of *Drosophila repleta* during continuous fixed flight. Each curve records the flight of a different individual. The flights were made at room temperature, constant within ± 0.1 C., as follows: No. 1, 25.4 C.; No. 2, 24.5 C.; No. 3, 24.8 C.

mounted specimens which had not undergone similar exertion lived upward of 36 hours (unfed) as a rule. The final rate-level lies at about 50–60 per cent of the starting-level in most cases. Marey (1869) states that, when his specimens had become fatigued after a long experiment, the rate fell to a fourth or a fifth of the previous figure. If the insects were then freed, their flight was slow and of short duration. He also found that in fresh specimens the rate might vary by as much as 3:1, and recorded such variation graphically. Magnan (1934) noted a reduction of $33\frac{1}{3}$ per cent in wing-rate during photographic experiments with *Macroglossa stellatarum*, a sphingid, and attributed it to fatigue. Other similar citations could be made if space permitted.

THE EFFECTS OF VARIATION IN ENVIRONMENTAL TEMPERATURE

With few proved exceptions insects are thought generally to be poikilothermic. The observations now to be detailed show that, in *D. repleta* also, wing-rate is very definitely under the influence of environmental temperature. The internal temperature may be

expected to have an even more direct effect, but it has not yet proved feasible to measure the internal temperature of such small insects during rate observations. The rate curves obtained permit the inference that internal temperature in this species parallels external temperature over the greater part of the effective range.

A series of random individuals of *D. repleta* was mounted, as above. The thermometer, graduated to 0.1 C., was fixed with the bulb 6 inches in front of the Strobotac. Flight was induced by lifting the mount with forceps to the level of the thermometer bulb and about 2 cm. to one side of it. Rate and temperature readings were taken simultaneously during the tenth second of flight. The specimens and apparatus were screened from air currents during the observations. Between 25° C. and the lower limit, temperature was varied by controlling the ventilation of the room. Observations above 25° C. were made by placing specimens and apparatus in a glass-topped wooden case. This was heated, as required, with a battery of electric lights. Armholes in the front of

TABLE 1
ADAPTATION OF *Drosophila repleta* TO CHANGING TEMPERATURE
DURING CONTINUOUS FLIGHT
(Specimen No. 9A 3/18/38)

Temperature (° C.)	Wing-Rate (Double Vibrations per Second)	Adaptation Time (Seconds)	ΔT (° C.)
27.3.....	213
17.4.....	175	17.0	9.9—
20.8.....	195	29.0	9.4+
17.6.....	173	29.0	9.2—
26.4.....	207	25.8	8.8+
17.6.....	168	24.2	8.8—
26.1.....	198	26.6	8.5+

the case allowed the necessary manipulations. The specimens were screened from the heating lights at all times, and the heating unit was turned off during observations. Humidity was not controlled, and excessive dryness may have contributed to the rapid incapacitation of specimens at temperatures slightly above 30° C. Intervals between readings on any one specimen were always longer than 1 minute and were frequently as long as several minutes. Possible artifacts were avoided by using a random, rather than a regular, succession of temperatures.

Since the rate at which the insects might adapt to a change in temperature was not known, experiments were run to test this point. The procedure was as above. Thermometers were placed both inside and outside the heated case. Flight was induced in the usual manner in either situation, and after the first rate reading the specimen was moved, while still in flight, to the atmosphere of different temperature. The length of time required to reach a new steady rate-level was measured with a stop watch. Typical results are recorded in Table 1. The response is reversible and may be reproduced *ad lib* with the same specimen, with results consistent within the usual range of variation. The data quoted show that a specimen in flight becomes adapted to a temperature change of the order of 10° C. within less than 30 seconds. The flight duration of 10 seconds ordinarily employed in the temperature experiments is ample for adaptation to

the small temperature differences between successive observations, a fact which was verified by measurements made after much longer exposures. It is thought likely that the greatly increased rate of respiration during flight (Demoll, 1927; Kalmus, 1929; Jongbloed and Wiersma, 1934) may account for the relatively rapid adaptation noted. It is believed that specimens at rest come to equilibrium with an atmosphere of changed temperature much more slowly.

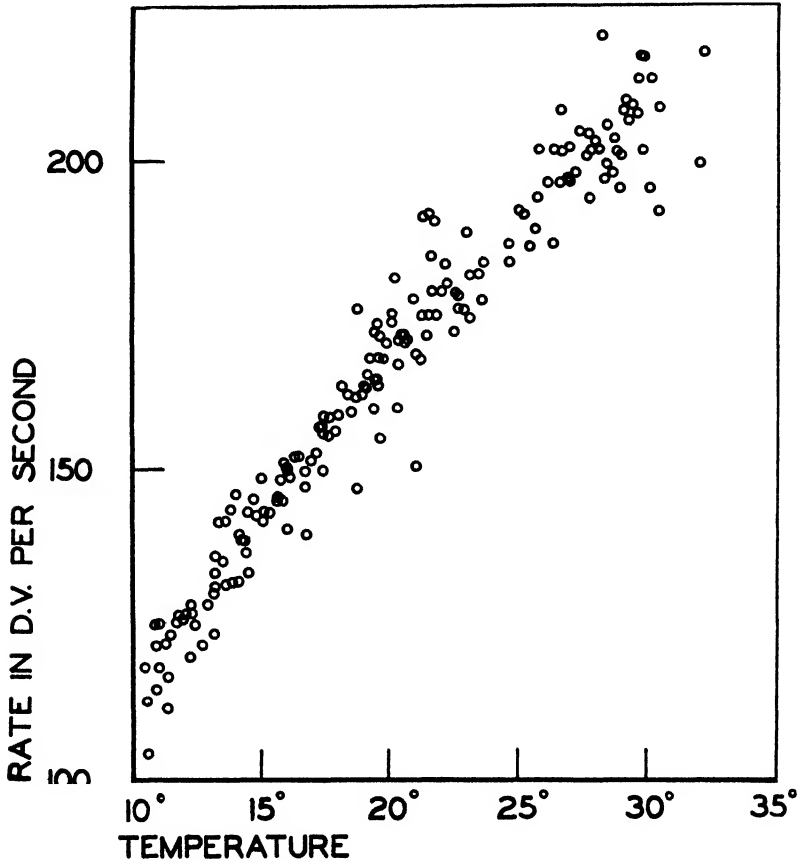


FIG. 2.—Variation in wing-rate of *Drosophila repleta* with varying temperature. Specimen No 12♂, 2/26-27/38.

A further reason for taking rate measurements only after flight has lasted some seconds is that, during the first few seconds, the rate reaches a value above the steady level presently adopted. This may be a reflex expression of the need for greater energy in the take-off than in maintaining flight. The changes involved follow one another too rapidly for accurate recording with the stroboscopic method but should be analyzable photographically.

The rate-temperature data were plotted for each specimen, as shown, for one example, in Figure 2. Observations were also averaged within 1° intervals (Table 2).

The absolute rate-level may be very different for different individuals (Table 3; cf. also Fig. 1). It is thought likely that metabolic age may be of importance in this connection, but the question remains to be tested. With the random selection of specimens which has been employed, sex and size show no correlation with the absolute rate-level. The slope of the rate-temperature curve is quite constant for all specimens, however; and hence it is considered permissible to average points determined on different indi-

TABLE 2
VARIATION IN WING-RATE OF *Drosophila repleta* WITH VARYING TEMPERATURE*
(Specimen No. 12♂ 2/26-27/38; cf. Figure 2)

<i>T</i> (°C.)	<i>R</i> (D.V./Sec.)	<i>D</i> (per Cent)	<i>n</i>	<i>T</i> (°C.)	<i>R</i> (D.V./Sec.)	<i>D</i> (per Cent)	<i>n</i>
10.7.....	118	11.4	9	22.6.....	179	5.1	8
11.7.....	122	8.6	8	23.4.....	180	2.9	5
12.4.....	125	4.2	7	24.7.....	188	2.3	3
13.5.....	136	9.1	11	25.5.....	193	4.9	5
14.6.....	140	6.1	12	26.6.....	199	5.9	9
15.6.....	146	4.1	11	27.7.....	200	3.3	7
16.5.....	149	5.9	7	28.6.....	202	8.7	10
17.5.....	156	4.1	10	29.3.....	210	3.9	9
18.6.....	162	9.4	9	30.3.....	202	5.5	4
19.5.....	167	6.9	15
20.5.....	169	11.0	12	32.2.....	208	4.4	2
21.5.....	180	6.7	10				

* *T* = average temperature; *R* = average rate; *D* = greatest deviation from average rate; *n* = number of observations.

TABLE 3
VARIATION IN WING-RATE OF DIFFERENT INDIVIDUALS OF
Drosophila repleta AT ONE TEMPERATURE LEVEL

Specimen No.	Average Temperature (°C.)	Average Rate (D.V./Sec.)	Number of Observations
12.....	20.5	169	12
18.....	20.6	193	4
19.....	20.5	200	7
1a.....	20.4	174	5
3b.....	20.6	160	7
7a.....	20.5	173	7

viduals in order to construct an average curve for the group. This is rendered necessary because of the impossibility of taking sufficient observations on one individual over the whole temperature range. Figure 3 shows such an average curve plotted from data (Table 4) on twelve individuals.

It should be noted that some real irregularities in the slope of the curve may have been obscured by the process of averaging. The data from numerous individuals agree in suggesting the presence of such irregularities at levels of about 15° C. and 22° C., in addition to the upper and lower limits and the optimum. In different individuals these

irregularities may be displaced slightly along the temperature axis, and therefore tend to disappear in the averages. The individual range of variation at any given temperature, together with the displacement just mentioned, makes it impossible at present to

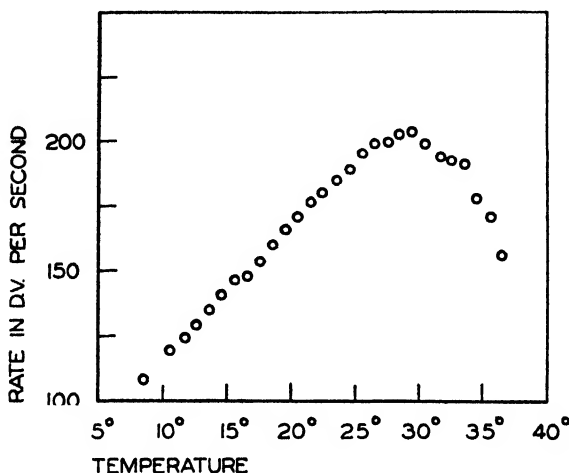


FIG. 3.—Variation in wing-rate of *Drosophila repleta* with varying temperature. The curve is a composite from twelve series of observations. Cf. Table 4 and text.

TABLE 4

VARIATION IN WING-RATE OF *Drosophila repleta* WITH VARYING TEMPERATURE
(Composite from Twelve Series of Observations; cf. Figure 3)

Average Temperature (°C.)	Average Rate (D.V./Sec.)	Number of Observations	Average Temperature (°C.)	Average Rate (D.V./Sec.)	Number of Observations
8.5.....	108	5	23.6.....	185	44
10.5.....	119	15	24.7.....	189	54
11.8.....	124	22	25.5.....	196	10
12.6.....	129	22	26.5.....	199	38
13.6.....	135	28	27.6.....	200	20
14.6.....	141	28	28.4.....	203	25
15.7.....	147	26	29.4.....	204	37
16.6.....	148	32	30.4.....	199	23
17.6.....	154	37	31.7.....	194	9
18.6.....	160	36	32.5.....	193	15
19.6.....	166	62	33.5.....	192	17
20.5.....	171	46	34.5.....	178	10
21.5.....	177	40	35.6.....	171	11
22.4.....	180	42	36.5.....	156	4

give any statistical confirmation of the existence of these "critical points." But other available facts indicate that it would be unwise to pass them by without further inquiry.

The break at 8°–10° C. is sudden. It has not been possible to induce flight movements at lower temperatures. However, once the starting-level is reached, vibration begins at

a rate of 90–100 per second rather than at zero. This fact strengthens a suspicion that the controlling mechanism lies outside the wing musculature.

The portion of the curve between the lower limit and about 15° C. is all an artifact, in a certain sense. Unmounted specimens could not be made to fly in this range. Only when they had been mounted and jerked into the air did the usual flight-releasing reflex come into play. It is to be doubted that these "flights" below 15° C. have any counterpart in nature. The general behavior below 15° C. is exceptionally docile, as compared with that at higher temperatures. Presumably some far-reaching physiological change takes place near this level.

Again in the neighborhood of 22° C. a less tangible but not dissimilar alteration in behavior occurs. General activity is suddenly heightened. Previously docile specimens all at once become unmanageable. Their power of co-ordination approaches that of more highly organized insects.

Once the optimum temperature has been exceeded, disintegration is rapid. Response is more irregular. One or two flights at 34° C. or above, and often at even lower temperatures, render previously vigorous specimens unfit for further experimentation. They become lethargic and appear unable to spread the wings for flight. Raising these flies from the support induces no more than a feeble effort toward opening the wings, which are, furthermore, carried abnormally and not tucked neatly back as usual. Unlike the changes previously discussed, this reaction is not reversible. Seldom does one of these specimens recover sufficiently, on removal to a lower temperature, to make a flight of even a few seconds' duration. For these reasons the shape of the curve above the optimum has been determined less satisfactorily than below this point.

These observations warn one against too facile an interpretation of the rate-temperature data. Particularly, they suggest that what is being measured here is something which affects the whole organism and not just the flight function, in terms of which the data have been gathered and expressed. At the same time, they justify the expectation that further quantitative studies on wing-rate and related aspects of flight will lead to information of general physiological significance. Should a population be found in which there is less variation in the response at one temperature-level, a favorable opportunity would be presented for the solution of some of the questions raised by this first investigation.

Finally, two additional points of interest may be noted. The average Q_{10} over the range 15°–25° C. is only 1.32. The data, in present form at least, do not fit the Arrhenius equation. In these respects the flight mechanism presents an interesting contrast with what previous workers—for example, Shapley (1920)—have observed in regard to other neuromuscular activities of insects (cf. also Crozier, 1924).

CONCLUSION

The factors for variation in rate which have been noted during these experiments may be classified arbitrarily in two groups on the basis of the magnitude of their effect. Certain factors, such as temperature and fatigue, have a far-reaching and lasting influence, the rate being alterable by as much as 50–60 per cent of its value at, say, 25° C. Other factors, such as sensory stimuli, seem rather to cause temporary fluctuations, within narrower limits, of the basic rate. The level of this basic rate is determined by processes which are unknown but which presumably affect the whole organism and

not only the flight function. It is likely that temperature is one factor which plays an important part in these processes. Fatigue may be supposed to have a more direct effect on the flight mechanism. Species, size, sex, metabolic age, seasonal variation, load, barometric pressure, and humidity are other variables, some of which have already been shown by other workers to have a bearing on wing-rate. Considering the number of these variables and the complexity of the combinations into which they may enter, it is evident that much remains to be done before any satisfactory interpretation of variation in wing-rate can be reached. It would therefore be premature to attempt a comparison of the results reported above with rate-temperature data, such as that accumulated by Crozier (1924), for other neuromuscular activities in insects. At the same time, it is obvious that rates determined without regard to these influences must be largely devoid of physiological significance.

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ACCLIMATIZATION OF FRESH-WATER CILIATES AND FLAGELLATES TO MEDIA OF HIGHER OSMOTIC PRESSURE¹

(Three figures)

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THE occurrence of closely related species of Protozoa in fresh and salt water is well known. Whether it be assumed that their ancestral prototype lived in a marine or in a fresh-water environment, one of the diverging groups has obviously become inured to a greatly changed osmotic pressure. Many fresh-water species are known to exhibit a wide tolerance to higher osmotic pressures. For example, Zeulzer (1907) had induced the fresh-water form *Amoeba verrucosa* to live in sea water by gradually increasing the concentration. Chatton and Tellier (1927a, 1927b) were able to acclimate *Glaucoma piriformis*, *G. scintillans*, *Colpidium colpoda*, *C. steini*, and *Paramecium caudatum* to concentrations of sodium chloride much higher than those in their normal environments. Berger (1929) reported that *Colpidium* was very resistant to changes in osmotic pressure and accordingly was unsuitable for a study of ion antagonism. Viewegerowa (1930) found that *C. colpoda* (?) could adapt itself to a saline solution somewhat less concentrated than sea water. And according to Finley (1930), the following series of forms could be acclimated to sea water: *Amoeba* sp. (*limax* type), *A. verrucosa*, *Bodo uncinatus*, *Colpoda aspera*, *Cyclidium citrullus*, *C. glaucoma*, *Euglena oxyuris*, *E. sp.*, *E. terricola*, *Euplotes charon*, *E. patella*, *Lionotus fasciola*, *Monas elongata*, *M. gelatinosa*, *Paramecium aurelia*, *P. caudatum*, *Phacus pleuronectes*, *Pleuromonas jaculans*, *Stylonychia pustulata*, and *Uronema marina*. Frisch (1935, 1937), however, reported that *P. caudatum* and *P. multimicronucleatum* always died when the salt concentration of the medium approached that of 40-45 per cent sea water. Yocom (1934) obtained flourishing cultures of *E. patella* in 65 per cent sea water, although *P. caudatum* and *Spirostomum ambiguum* were less adaptable. Furthermore, marine protozoa can withstand osmotic pressures lower and higher than those of sea water (cf. Schaeffer, 1926). Butts (1935) observed that the marine amoeba *Flabellula* could reproduce in artificial sea water ranging from 20 to 150 per cent, although reproduction in concentrations below 20 per cent was rare. The optimum for growth was 90 per cent sea water, 10 per cent below that of the normal environment. More recently Hopkins (1938) reported that *Flabellula mira* can withstand direct transfer from 100 to 5 per cent sea water, or vice versa.

The extent to which different species possess an osmotic tolerance is of evolutionary significance, for, if acclimatization is demonstrable, the result is a clear case of environmental modification of certain physiological regulatory mechanisms. Tolerance for a higher or lower osmotic pressure may well have been a vital factor in the survival of protozoan species and may at some future time again be vitally important to the species.

¹ Grateful acknowledgment is made to the Kentucky Academy of Sciences for an award for purchase of certain materials and equipment, and to Dr. R. P. Hall for reading the manuscript.

Perhaps it was their degree of osmotic adaptability which originally permitted divergence of Protozoa into salt-water and fresh-water environments.

In order to obtain more information on this problem, which is recurrent in the literature but has received relatively little experimental attention, comparative experiments were undertaken with bacteria-free strains of *Colpidium campylum*, *G. piriformis*, *Euglena gracilis*, *Chlorogonium euchlorum*, and *Astasia* sp. The effects of sudden and of gradual changes to higher salt concentrations were determined, as well as the effects of change from high to lower concentrations.

MATERIAL AND METHODS

Bacteria-free strains of *E. gracilis* and *C. euchlorum* were obtained originally from Professor E. G. Pringsheim, and the strain of *Astasia* sp. was isolated by Dr. T. L. Jahn. The strains of *C. campylum* and *G. piriformis* are the ones used by Elliott (1935) and Johnson (1935), respectively.

Medium "B" (Loefer, 1936) was used for stock cultures. Its composition was as follows: NaCl, 0.003 gm.; CaSO₄, 0.015 gm.; MgSO₄, 0.0045 gm.; KNO₃, 0.0013 gm.; FeCl₃, 0.0002 gm.; distilled water to 1 liter.

The experimental media were prepared by adding Bacto-tryptone (0.2 per cent) and Difco Yeast Extract (0.01 per cent) to Van't Hoff's solution, concentrated (100 per cent) and in various dilutions (1 per cent, 5 per cent, and in successive 5 per cent grades to 95 per cent). The Van't Hoff solution, prepared according to the formula used by Hall (1925), contained 5/8 M solutions of the following salts mixed in the proportions indicated: CaCl₂, 1 cc.; MgCl₂, 7.8 cc.; MgSO₄, 3.8 cc.; KCl, 2.2 cc. Each medium was tubed in 10-cc. amounts and sterilized in the autoclave. Hydrogen-ion concentration in all cases was pH 6.7; incubation was at room temperature (26°-31° C.).

A preliminary experiment was performed in order to determine whether a 1 per cent Van't Hoff medium would support growth of the five species listed above. Inocula of several drops were used for seeding successive subcultures at 3-day intervals. Since all the species were flourishing after nine successive transfers, the medium was considered adequate for maintenance of each species and hence suitable for investigating the effects of different salt concentrations.

The technique used is similar to that employed in earlier investigations (Loefer, 1934, 1935). In carrying out the experiments an effort was made to acclimatize the organisms by allowing them to live for several generations in a given solution and subsequently transferring them to gradually increasing concentrations of saline. Motility, as determined by direct observation of a milliliter sample under a binocular, and viability were the criteria for tolerance.

EXPERIMENTAL RESULTS

SERIES I. *Colpidium campylum*, *Glaucoma piriformis*, *Euglena gracilis*, *Astasia* sp.

Van't Hoff media were made up with salt concentrations of 1, 5, 10, . . . , 80 per cent for the first five transfers. In later transfers the concentrations were extended to 100 per cent. After sterilization several tubes at each degree of salinity received a 3-drop inoculum of one of the four species in 24-48-hour cultures in the stock medium. This procedure was repeated for each species. All tubes of the first transfer were incubated at room temperature (26°-31° C.) in moderate light for 24 hours and were then examined

under a binocular for living organisms. If no motile organisms were observed, a viability test was made by transferring 1 cc. of the contents of the experimental tube to a tube of stock medium. The results recorded in Figure 1 designate the highest concentration from which viable organisms were obtained. In all cases the organisms were viable from the lower concentrations tested. Thus, in the first transfer *Astasia* sp. remained viable in all tubes up to and including 80 per cent Van't Hoff solution, *E. gracilis* in all up to and including 35 per cent, and so on.

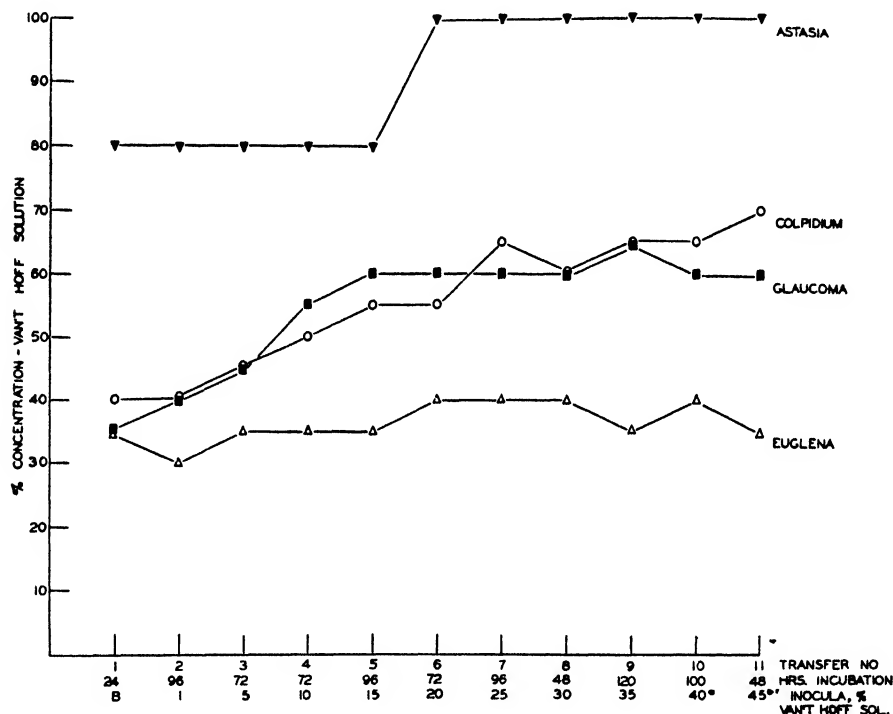


FIG. 1.—Series I. *Astasia* sp., *Colpidium campylum*, *Glaucoma piriformis*, and *Euglena gracilis*. Symbols represent upper limits of viability in each transfer. Asterisk (*) indicates that *E. gracilis* cultures were seeded from a 35 per cent Van't Hoff solution stock; prime (') indicates that *Astasia* cultures were seeded from a 40 per cent Van't Hoff solution stock.

In the second transfer the media of different salt concentrations were inoculated from a first-transfer tube of each species containing 1 per cent Van't Hoff solution. In the third transfer a second-transfer tube, containing 5 per cent salt solution, was used for inoculations. In subsequent transfers the tubes used for inoculation contained 10 per cent, 15 per cent, 20 per cent, . . . , 45 per cent salt solution, respectively, the latter concentration being used for the two ciliates in the eleventh transfer. The maximal salt concentrations in which the organisms remained viable in the eleven transfers are indicated in Figure 1, which indicates also the incubation time and the stock medium used for each transfer.

Euglena gracilis showed no definite indication of acclimatization to higher salt con-

centrations, viability being observed only within the range 1-40 per cent; and there was no evidence of an increased tolerance in later transfers. The 40 per cent solutions which they withstood represent a total salinity of 1.59 per cent. The organisms were carried for several subcultures in this concentration, but even this longer period in the near critical concentration seemed to have no effect on tolerance. *Astasia* sp. remained viable in 80 per cent Van't Hoff medium in the first five transfers and in 100 per cent in the last six transfers. Since the higher salt concentrations were not tested in the first five transfers, it was impossible to determine whether this species had shown any definite acclimatization or was merely adaptable to a wide range of salt concentrations. *Colpidium campylum* showed a definite increase in salt tolerance in the later transfers, the limit being increased from 40 per cent in the first and second transfers to 70 per cent in the eleventh transfer. The behavior of *G. piriformis* was comparable to that of *C. campylum*, the former remaining viable in 60 per cent Van't Hoff medium in the eleventh transfer.

SERIES II. *Colpidium campylum*, *Glaucoma piriformis*, *Chlorogonium euchlorum*, *Astasia* sp.

In this series, which was carried for thirteen to fifteen transfers, the higher, as well as the lower, salt concentrations were used in each transfer. The results are recorded in Figures 2 and 3. It will be noted that the technique differed from that of Series I with respect to the cultures used for inoculation. Thus, in the second transfer of *C. campylum* (Fig. 2), a 20 per cent Van't Hoff culture from the first transfer was used for inocula; in the third transfer, a 25 per cent culture from the second transfer; and in the fifteenth, a 90 per cent culture from the fourteenth transfer. At the last transfer a companion series of tubes (15A) was also inoculated from a culture in stock medium as an additional check on the progress of acclimatization. Figure 3 shows the results on the flagellates used in this series. They were carried through thirteen transfers when a companion check series (13A) was also incubated. Periods of incubation were identical for the two ciliates, except as indicated in Figure 2 for transfers 15 and 15A; incubation times were identical for the two flagellates, as seen in Figure 3. It will be observed from Figures 2 and 3 that viability was noted in salt concentrations higher than those in which the organisms remained motile. This difference was striking in the case of *Astasia* sp., which never remained motile in salt concentrations above 40 per cent.

The results showed acclimatization of *C. campylum* to 95 per cent Van't Hoff solution (3.78 per cent total salinity), of *G. piriformis* to 70 per cent solution (2.78 per cent total salinity), of *C. euchlorum* to 65 per cent solution (2.58 per cent total salinity). These results are significant changes. Thus, on the first transfer from the stock medium, *Colpidium* could not tolerate more than a 30-40 per cent Van't Hoff solution (1.2-1.6 per cent salinity). Acclimatization is also clearly shown by a comparison of transfers 15 and 15A. Whereas the acclimated strain of transfer 15 easily tolerated the higher salinities, the stock strain (transfer 15A) showed the same osmotic tolerance as at the beginning of the experiment (transfer 1). The negative results with all cultures of the third transfer of *C. campylum* and *G. piriformis* were the result of accidental exposure of the cultures to direct sunlight. In no case did transfer of the ciliates from higher salinities back to the lower salinities result in a loss of viability, and in this respect the results are in complete agreement with those of Series I. *Colpidium campylum*, as well as other species tested, must be subcultured frequently in the media of higher salt concentration.

The limit of motility for *G. piriformis* was a 65 per cent solution; and the limit of

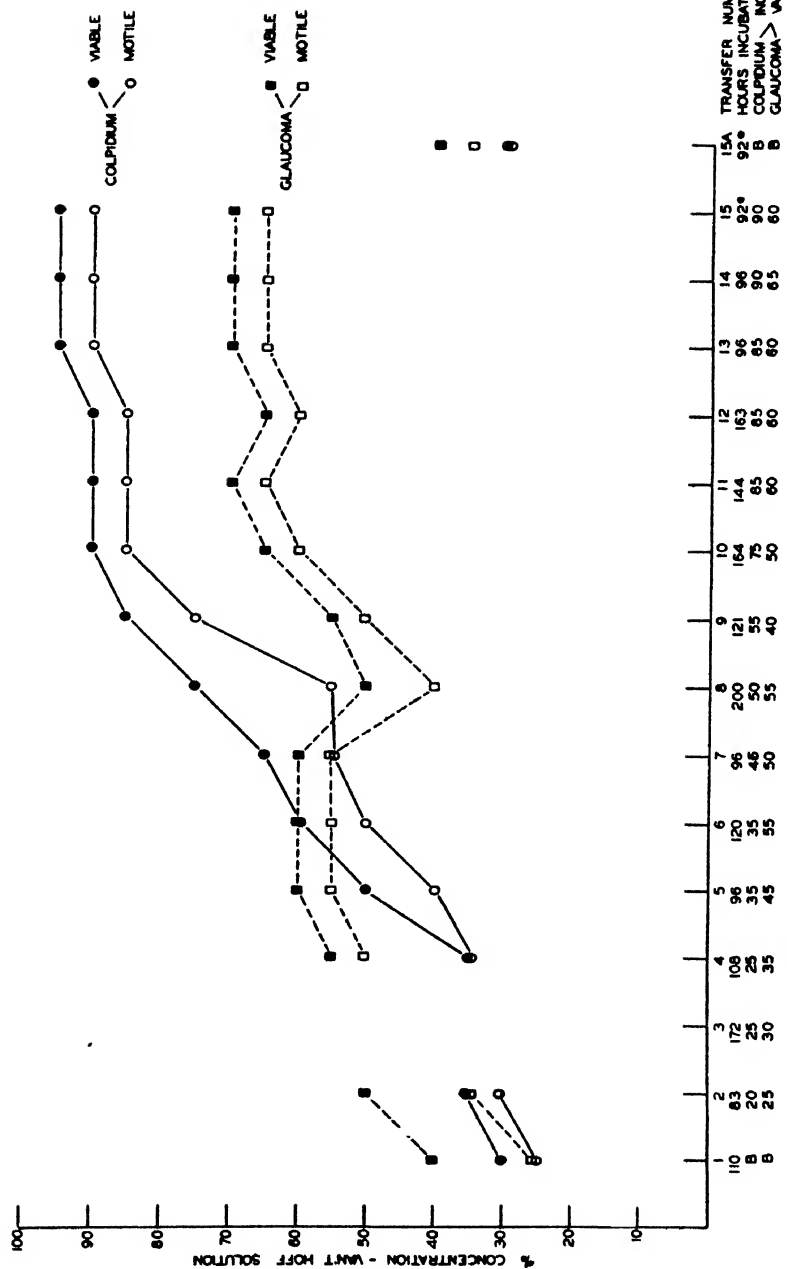


FIG. 2.—Series II. *Colpidium campyllum* and *Glaucocoma piriformis*. Symbols represent upper limits of motility and viability in each transfer. Asterisk (*) = 96 hours for *Glaucocoma*.

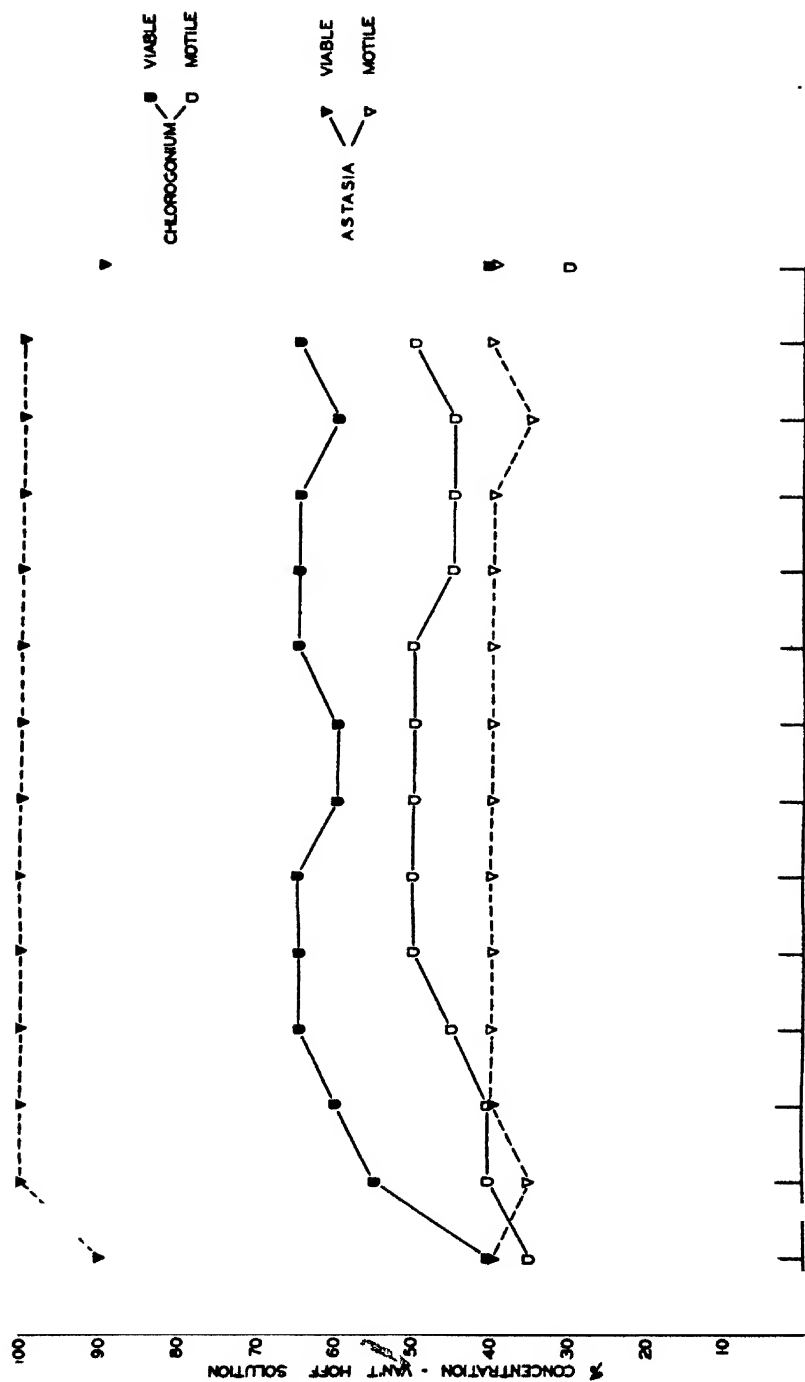


Fig 3.—Series II. *Chlorogonium euehlorum* and *Astasia* sp. Symbols represent upper limits of motility and viability in each transfer

viability, a 70 per cent Van't Hoff solution. This species, therefore, withstood a total salinity of about 2.78 per cent after eleven to fifteen transfers. Comparison of transfers 1 and 15A, inoculated from cultures in stock media, with transfer 15 indicates the amount of developed osmotic tolerance.

In the first transfer *Chlorogonium* was motile in 35 per cent Van't Hoff solution and viable in 40 per cent solution after 110 hours (Fig. 3); essentially similar results were obtained in transfer 13A, also inoculated from a stock culture. In transfers 2-13, however, the organisms withstood greater salinities and in the later transfers survived for as long as 200 hours in a 60 per cent Van't Hoff solution (total salinity of 2.38 per cent). This indicates a definite adaptation to higher osmotic pressures approaching in degree that acquired by *Glaucoma*.

The results obtained with *Astasia* were unusual, since, regardless of the salt concentration of the inoculum, the flagellates were never motile in any of the transfers in concentrations of Van't Hoff solution above 40 per cent (total salinity, 1.59 per cent). In transfers 1 and 13A viability was apparently lost upon transfer from cultures in stock medium to 95 per cent and 100 per cent Van't Hoff media, but in all other transfers the flagellates remained viable in 100 per cent Van't Hoff solution. In this respect *Astasia* sp. differs from the other species tested. Furthermore, the increase in tolerance was negligible in the case of *Astasia*, which apparently possesses a very high degree of normal tolerance to high salt concentrations.

MORPHOLOGICAL CHANGES IN THE CILIATES

The organisms in the solutions with higher salt contents appeared to differ considerably from the original stock. This was especially true of the ciliates, which were no longer recognizable as belonging to the same species. Thus, *Glaucoma* from the 1 per cent dilutions averaged 50 μ in length, but only 42 μ in the 35 per cent solutions. Their contractile vacuoles were no longer apparent in the higher salinities. In concentrations near the vital limit, irregularities in shape, such as may be observed in old cultures, were apparent after several days.

DISCUSSION

From the results described above, it is obvious that *C. euchlorum*, *G. piriformis*, and *C. campylum* gradually developed a tolerance to hypertonic solutions during a series of transfers into more and more concentrated solutions of artificial sea water (Van't Hoff solution). The species differed in the degree of their acclimatization, *Colpidium* having developed a greater osmotic tolerance than the others. The total salinity of the 100 per cent Van't Hoff solution used in these experiments is equal to 3.97 per cent and, therefore, is greater than that of the North Atlantic Ocean, for which Henderson (1927, p. 173) gives a total salinity of 3.2-3.3 per cent. An 85 per cent Van't Hoff solution would contain salts in this concentration. Hence, it is apparent that only *Colpidium* could adapt itself to an osmotic pressure equivalent to that of sea water under the conditions of the experiments. While *Astasia* remained viable in higher salt concentrations, motility was always lost above 40 per cent Van't Hoff solution.

The results on *C. campylum* are in agreement with the general report of Berger (1929). In their experiments with monobacterial cultures of *C. colpoda* and *C. steini*, Chatton and Tellier (1927a) found a "distal limit" of 1.0 per cent NaCl, considerably lower than the concentration tolerated by *C. campylum* in these experiments. Finley

(1930) gives further data on *C. colpoda*, indicating a small increased tolerance—from 15 to 20 per cent sea water. It appears that *C. campylum* is more tolerant than either *C. colpoda* or *C. steini*. Viewegerowa (1930) reported also a definite, although temporary, acclimatization of this species. From the rapid division-rate indicated (seven to eight divisions in 24 hours), it seems likely that *C. campylum* or some other fast-growing species was used by Viewegerowa instead of *C. colpoda*.

Glaucoma scintillans, according to Finley (1930), increased its tolerance from 7 to 20 per cent sea water, whereas *G. piriformis* in the present experiments developed a resistance to 70 per cent Van't Hoff solution from a natural tolerance of 40 per cent. As in the genus *Colpidium*, the foregoing appears to be a marked species difference. The highest concentration of NaCl which *G. scintillans* could withstand, according to Chatton and Tellier (1927a), was 1.3 per cent; while *G. piriformis* could tolerate 1.8 per cent, a concentration above the vital limit of the other four ciliates they tested. Yet even this is considerably less than the total salt concentration (2.78 per cent) withstood by *G. piriformis* in the present experiments. After the second transfer the ciliate withstood a concentration equivalent to Chatton and Tellier's "distal" limit for this organism.

While the results on *Colpidium* and *Glaucoma* in the present experiments with bacteria-free cultures are not strictly comparable to those of previous investigators, it is interesting to note that the degree of tolerance of these two ciliates resembles that of *C. citrullus*, *C. glaucoma*, *E. charon*, *E. patella*, *L. fasciola*, *P. caudatum*, *P. aurelia*, *S. pustulata*, and *U. marina*, according to Finley's (1930) list of thirty-one ciliated species. Frisch (1935, 1937), however, could not verify the high tolerance of *P. caudatum*. He states (1937): "... many individuals survived until a concentration of 45% was reached." This would be equal to a concentration of about 1.5 per cent saline, which is, however, considerably greater than the 1 per cent "distal limit" for *P. caudatum* reported by Chatton and Tellier (1927a).

The adaptability of the genus *Colpidium* to higher osmotic pressures may explain its wide distribution in salt and fresh water. If *Colpidium* is capable of inuring itself to the tonicity of sea water in the course of relatively few generations under experimental conditions, the same thing may have occurred under natural conditions in repeated instances. The generation time of this organism is only a few hours, and the actual number of generations produced in these experiments may be estimated. The total number of incubation hours up to the tenth transfer was 1,106 (1,861 hours through fifteen transfers). If an average generation time of 11 hours is estimated, there would have been 100 generations up to the tenth transfer (169 after fifteen transfers). Environment appears to be the immediate causative factor for this acclimatization; hence, factors of use and disuse or modification of certain physiological regulatory mechanisms are involved, and the inheritance of at least temporarily acquired characters seems clearly demonstrated. The problem confronting the organisms was a vital one, permitting only two alternatives—to develop an osmotic tolerance or die. During the estimated 169 generations most of the organisms no doubt succumbed, although the few that had better regulatory mechanisms lived and multiplied. A similar selective survival method is postulated by Hall and Schoenborn (1939) for the effect of certain nutritive substances on *Euglena*. The duration of these experiments was relatively short; and it is not unreasonable to believe that if *Glaucoma* and *Chlorogonium* had been subjected to finer gradations of tonicity over longer periods of time, they, too, would have shown greater acclimatization. At any rate, they did exhibit an appreciable degree of flexibility in the course of

fifteen transfers. This idea would seem to be at variance with that expressed by Chatton and Tellier (1927a, 1927b), who contend that it is not necessary to acclimate an organism to higher salt concentration by degrees. Yet it is evident from the present experiments that viability in the higher salinities was not attained unless the stock was already growing in a relatively highly concentrated saline.

Although the tolerance which the flagellates developed was not as great as that of the ciliates, *Chlorogonium* acquired about as much as *Glaucoma*. *Euglena gracilis* apparently acquired none. Although viable in a few instances from a 40 per cent dilution, its motility limit appeared to be 35 per cent. It withstood almost this concentration on sudden transfer. Hence, the salinity tolerated on direct transfer is similar to that given by Finley (1930) for three species of *Euglena*. He found, however, that all three became adapted to solutions of 103 per cent sea water. He states (p. 345): "Non-flagellate motionless stages of Euglenae were frequently seen in the high concentrations of sea water, and many Euglenae were actively swimming in concentrations of 103 per cent sea water." The viabilities recorded for *E. gracilis* up to 40 per cent Van't Hoff solution in Figure 1 were in many cases from nonmotile cultures. Only three of thirteen flagellated species which Finley tested failed to develop tolerance to solutions up to 80 per cent sea water. Hence, the proportion of flagellates which became acclimated is considerably higher than for the ciliates, where twenty-one out of thirty-one developed a tolerance no greater than to 52 per cent sea water. The motility limit of *Astasia* in the present series was 40 per cent, but these organisms were generally viable from all concentrations up to 100 per cent. If we consider viability as a criterion, this unusual organism is euryhaline, but not so if motility is our criterion. Whether the immotile forms represent true cysts is doubtful, since they are not rounded up in the usual manner. Wolff (1927a) observed failure of normal encystation of *Hartmanella* in 3-4 per cent NaCl and describes the process simply as a rounding-up. Such a mechanism could conceivably be of great value in the distribution of species, provided they were finally returned to a favorable environment. In fact, it may represent nothing more than a degree of cyst formation.

The unusual behavior just considered is but one of several environmental effects on morphology. There seems to be a diversity of opinion concerning induced morphological changes in protozoan organisms. As far as these experiments are concerned, the ciliates were most strikingly affected. *Glaucoma* from the 1 per cent solutions averaged 50 μ in length, but only 42 μ in the 35 per cent solutions. Their contractile vacuoles were no longer apparent in the higher tonicities. No slowing of the rate of ciliary movement was observed. These observations are in general agreement with the reports of earlier investigators (Zeulzer, 1907; Wolff, 1927b; Kitching, 1934) from their studies dealing with the nature and function of the contractile vacuole. Finley (1930, p. 343) observed in the case of *A. verrucosa* that

in 84% sea water the contractile vacuole was not present. . . . The Amoebas were rather active in concentrations as high as 44 per cent sea water, but above that they moved slowly.

The case of the Amoebas was exceptional, for in the other protozoa observed no significant change in morphology occurred until it was apparent that the animal was on the verge of dying. In all the ciliates listed in Table I the pulsation of the contractile vacuole was relatively slower in high concentrations of sea water, but in no case other than *Amoeba verrucosa*, did the vacuole or its pulsation disappear entirely. . . .

As the salinity increased there was a very noticeable increase in the visibility of locomotor organelles; flagella and cilia were seen with much less difficulty in sea water.

Frisch (1935, 1937) reported decreased size and fewer vacuole pulsations in *Paramecium*; but he interprets these variations as due to decreased food and toxic effects of the salts rather than to direct results of osmotic pressure changes. In the present experiments soluble food for the ciliates was probably present in abundance, considering the general appearance of the controls; yet morphological changes in the higher salt concentrations were appreciable. It might be expected that the contractile vacuole would tend to disappear when its regulatory function became less important, i.e., when the osmotic pressure of the environment approached that of the cell itself. Gaw (1936) observed a much slower pulsation-rate in the contractile vacuoles of certain ciliates when the osmotic pressure of the medium was increased. In accordance with this idea is the finding of Hayes (1930) that the oxygen consumption of *Paramecium* decreased with increasing salinity, which indicates that less energy is required for regulation.

The size differences are of taxonomic interest, for they raise the question of what constitutes a species. Without doubt we had the same organism at the beginning of the experiment; but if the acclimated and normal types of *Glaucoma* had been independently studied from nature, in all probability they would have been considered different species. Such observations make it apparent that consideration must be given to physiological, as well as morphological, details before species can be accurately identified. *Glaucoma piriformis* and *C. campylum* may not be strikingly different in morphology, but they differ considerably in their physiological characteristics. The two forms exhibit different osmotic tolerances, different growth-rates, and different rates of dextrose consumption (Loefer, 1937, 1938b). The problem of species delimitation is still, however, a difficult one if organisms exhibit not only physiological but also morphological adaptations when grown in different media. Athanassopoulos (1931) observed such variations in several plankton forms and attributed them to a lessened salinity of the sea water. Morphological changes in the present experiments were, likewise, correlated with salinity changes. Other conditions may cause structural variations; e.g., Lefèvre (1931) reported that changes in hydrogen-ion concentrations caused variation in cellular metabolism as well as in cellular morphology. Changes were also noted and attributed to the hydrogen-ion concentration by Loefer (1938a).

The acquired osmotic tolerance of *Colpidium* and *Glaucoma*, however, did not prevent direct successful return to the original medium; and after the change was made, the organisms were again normal in size and appearance after several days. Chatton and Tellier (1927a) also observed that *Glaucoma* could be transferred suddenly from a hypertonic to a salt-free medium with no harmful effects, even though it had been growing in a solution near its vital limit for six months. Viewegerowa (1930), too, found that acclimated organisms, when returned to natural medium, soon lost their acquired resistance and in many cases could no longer withstand saline concentrations which were not harmful previously.

SUMMARY

Experiments were performed in an attempt to acclimatize five species of fresh-water protozoa, viz., *C. campylum*, *G. piriformis*, *C. euchlorum*, *E. gracilis*, and *Astasia* sp., to artificial sea water. The culture medium consisted of various dilutions of a Van't Hoff solution to which 0.2 per cent Bacto-tryptone and 0.01 per cent Difco Yeast Extract had been added. After eleven to fifteen transfers, in which stocks from increasingly concentrated salinities were used, *Astasia* was never motile above a 40 per cent dilution; it was

viable, however, even after 200 hours' exposure to concentrated Van't Hoff solution (100 per cent). *Euglena* developed no appreciable tolerance (40 per cent) but withstood 35 per cent on direct transfer. *Chlorogonium* adapted its viability from a normal of 40 to 65 per cent. *Glaucoma*, at first viable from 40 per cent, was finally viable from a 70 per cent solution. The acclimatization of *Colpidium* was greater than any of the other species. Its tolerance increased from a 30 to a 95 per cent Van't Hoff solution. The salinity of the latter is higher than that of ordinary sea water. All organisms, except perhaps *Astasia*, developed their tolerance only gradually through a number of generations. The transfer from the concentrated solutions back to the lower salinities could be made without fatal results.

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A MICROPOPULATION STUDY OF EUGLENA GRACILIS KLEBS IN STERILE, AUTOTROPHIC MEDIA AND IN BACTERIAL SUSPENSIONS¹

(Five figures)

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I. INTRODUCTION

ROBERTSON'S hypothesis of allelocatalysis has prompted a number of experimentalists to check the relations of numbers of organisms, volumes, and rates of division in order to test his proposition that "there is acceleration of multiplication by the contiguity of a second organism in a restricted volume of nutrient medium" (Robertson, 1924, p. 1242). This may be called the "Robertson effect." Robertson's hypothesis to account for this type of acceleration has been summarized clearly by Allee (1931, p. 166):

During nuclear division each nucleus retains the charge of autocatalyst with which it was provided, and adds to it during the course of nuclear synthesis. At each division the autocatalyst is shared between the nuclear substance and the surrounding medium in a proportion determined by its relative solubility and by its affinity for chemical substances within the nucleus. The mutually accelerative or allelocatalytic effect of contiguous cells is due to each cell's losing less of the autocatalyst to the medium because of the presence of the other.

Robertson's observations suggest some of the interrelationships existing between members of the same species of relatively asocial animals and plants which are known to affect the structural and functional characteristics of such individuals and even the integration of their communities. Tests of the reality of his theory have been made primarily with protozoans.

The work of several careful experimentalists has given evidence to support Robertson's phenomenon, if not his hypothesis, although even more work with numerous species of protozoans under various conditions tends to uphold the findings of Woodruff (1911), which are exactly opposite. These studies, both for and against the Robertson effect, are thoroughly reviewed by Allee (1931, 1934) and by Johnson (1937). Johnson (1936), whose work with *Oxytricha* and *Paramecium* has included the relations between reproductive rates of different numbers of individuals seeded in different volumes and varying densities of bacterial suspensions, has shown, among other things, that in the presence of sparse suspensions of the bacterium *Pseudomonas fluorescens*, cultures seeded with isolated individuals reproduced faster; with dense suspensions those seeded with two individuals showed greater reproductive rates. Gause (1934) terms this the "Johnson effect" and produces independent evidence in its support.

The relation and interrelation between numbers, food, and volume for each species of

¹ I am gratefully indebted to Dr. W. C. Allee, who suggested this problem to me and whose kindly advice and untiring interest have made the completion of this paper possible after an extended data-finding period and whose help in evaluating and clarifying the results has been invaluable.

animal and plant present in a microcosm is a complex problem, particularly when bacteria and other microorganisms are involved. Johnson (1937) also summarizes much of this work.

These experiments indicate that the number of individuals with which a culture is inoculated is not an absolute factor by means of which the ensuing division-rates of ciliates may be predicted but that with different species, in certain volumes and conditions, seeding a culture with greater numbers insures a faster initial division-rate, and vice versa; the greater numbers generally show relatively greater division-rates in larger volumes and/or when the bacterial content is high.

Such results resemble those which Robertson discovered and assigned to the allelo-catalytic effect of a nuclear X-substance given off into the medium during cell division, but these later results can be accounted for in terms of other quantitative relations which exist between the medium and the special organisms studied. There is a recent revival of the original theory in the reports of Karl Reich (1938) with an amoeba, *Mayorella palestinensis*, and of Mast and Pace (1938), with *Chilomonas paramecium*, in which the observed results apparently cannot be attributed wholly to nutrient-organism relations.

Much work has been done on the laboratory ecology of *Euglena gracilis*, which I shall not cite here, in view of the fact that there are extensive reviews and bibliographies to be found in such papers as those of Jahn (1934, 1935a), Dusi (1930, 1936), Mainx (1928), Zumstein (1900), and Dangeard (1901).

Since *E. gracilis* is an autotroph, there is opportunity, by limiting the bacteria, to check the effects of numbers of the same species in specific media more closely than with the ciliates or with other heterotrophic organisms.

Experimental work varying the numbers of *E. gracilis* in the inoculum has been attempted only by Jahn (1929, 1930). In a few isolation cultures he found no consistency in division-rates in small volumes. He attempted 2- and 6-drop cultures (size of drop not stated), which were inoculated with single individuals after two washings, details of which are not given. They were followed over a 10-day period. As a result of this experience he concluded that this method was impractical, owing to changes in salt concentrations from evaporation of small volumes over such a period of time and because of surface-interior relations which are not identical with those of cultures with larger volumes.

In mass cultures Jahn (1929) did not obtain absolutely sterile conditions but considered an initial bacterial count of 3,000 per cubic centimeter and a final count of 3,000,000 per cubic centimeter an inappreciable contamination. This number, however, may be responsible, at least in part, for the shape of his growth curves.

With one *Euglena* in from 383,300 to 353,300,000 times its volume of culture media, Jahn found that (1) division-rates decrease as the number of seeded organisms increases; (2) the division-rates decrease as time increases; and (3) the number of organisms increases in time and then decreases. As Jahn says (1930), an increase in numbers, taken alone, is not necessarily a test of an autocatalytic effect in a long-time study (cf. Morgan [1926] and Snell [1929]). Deaths aside, in counts taken at the end of the first 24 hours, however, the division-rate is indicated fairly directly by the number of individuals present.

Jahn (1929) found from mass cultures that the smaller the initial number of organisms per volume, the greater the numbers produced at the end of a 6- or 10-day period. In 48 hours the difference was not great but was slightly in favor of those cul-

tures inoculated with the greatest number of individuals. Without more information one can point out only that this acceleration in the second 24-hour period is what might be expected from either the Robertson or the Johnson effects, so called.

The present paper attempts to explore further the problem of interorganismic effects on the division-rate of *E. gracilis*. Experimental conditions include various number and volume relations, depression-slide cultures, mass flask cultures (not discussed here), relatively sterile cultures employing dilution and irradiation methods, and cultures in controlled bacterial suspensions. The experiments have been made to test the role of numbers of the same species and of controlled populations of associated bacteria with respect to specific volume relations as factors determining division-rates.

Preliminary experiments, using various techniques and types of infusion, showed no consistent results with reference to number in the inocula. These cultures contained such a large number of variables that an attempt was made to set up a series of systems in which the environmental conditions were more nearly under control.

The effects of different numbers in the inocula are mainly investigated, although certain cross-comparisons between growth in different cultures will be given. In this paper the following aspects of the problem are discussed:

1. A comparison of division-rates of cultures inoculated with one, two, four, and eight individuals in several small volumes, using dilution methods for obtaining relatively bacteria-free euglenae.
2. A similar comparison when the euglenae are freed from bacteria by exposure to ultra-violet irradiation.
3. Similar comparisons in suspensions of controlled concentrations of mixed or pure cultures of bacteria when the euglenae are freed from bacteria by both of the foregoing methods.
4. The effects of numbers present on survival after long exposure to ultra-violet irradiation.
5. A comparison of the results with these small-volume cultures with those of Jahn's mass cultures.

II. CULTURE METHODS

A clone of *E. gracilis* was isolated at the beginning of the summer of 1934 from a culture obtained from the General Biological Supply House, Chicago. The first clone served for all the cultures of 1934 and 1935. A second clone, established in the spring of 1936 and used for work of that summer, was found to be no different statistically in division-rates or any other measured characteristic from Clone I. This clone was isolated from a culture furnished by the Albert Galigher Laboratories of Berkeley, California.

The standard experimental medium throughout was the salt solution of Osterhout (1906), as modified by Barker and Taylor (1931), made with block-tin and then glass-distilled water and using analyzed grades of the following salts in a liter of water: 7.8 gm. $\text{MgCl}_2 \cdot \text{H}_2\text{O}$; 3.8 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2.2 gm. KCl ; 1.0 gm. CaCl_2 ; and 1.0 gm. NaCl . This was diluted 500 times with double-distilled water before autoclaving and using. One cubic centimeter of $\text{M}/20 \text{ NaH}_2\text{PO}_4$ was used to buffer each 30 cc. of the distilled solution, and pH adjustments were made with $\text{M}/20 \text{ NaOH}$.

Ferric chloride was added to each liter of the salt solution before using it, so that there was a concentration of 0.0002 per cent ferric chloride per liter. Unlike usual practice in flagellate culturing, no acetate, citrate salts, or organic material was used, since a medium

containing as little organic matter as possible was desired. Glucose, Leibig's extract, Difco tryptophane, peptone, or muscle extracts are added by most recent experimentalists, such as Jahn (1929-35), Alexander (1931), Hall (1933), and Elliott (1938). While several students of chlorophyll-bearing organisms, such as Zumstein (1900) and Ternetz (1912), reported unfavorable growth in an autotrophic medium, Pringsheim (1912) and Mainx (1928), working with both heterotrophic and autotrophic media, conclude that growth of flagellates was as good in an autotrophic as in a heterotrophic medium. They do not, however, present evidence concerning the degree of bacteriological sterility of their media. Jahn used the inorganic salt media of Mainx (1928) and Gunther (1928), to which he added hydrolyzed casein for most of his work. This salt solution also had either ammonium or potassium nitrate salts as nitrogen sources. Dusi (1933) succeeded in culturing several species of *Euglena*, using inorganic sources of nitrogen, and later (1936, 1937) carried through four transfers, at least, in a totally inorganic medium. Dusi also finds that growth is much facilitated when organic materials are present. Mast, Pace, and Mast (1936) report success in growing *Chilomonas*, a supposed saprozoite, on an inorganic medium, while Hall and Loefer (1936) failed to grow this species when the dilution of tryptone was beyond 1:100,000.

The solution used in the present work lacks nitrogen in quantities determinable by sulphanilic nitrite or Nessler's ammonium tests. However, the reagents used have minute quantities of nitrogen present as impurities; and, likewise, the distilled water shows traces of it. The question of nitrogen-fixation on the part of *Euglena* is entirely problematical. There was no evidence from tests that the nitrogen content increased in cultures in sterile salt solution in a 24-hour period. It is possible that sufficient nitrogen is stored within each organism to supply several generations, or the disintegration of one or more of the original individuals in a culture may furnish enough of this substance. The amount of organic stuff in a 1:500,000 dilution of the stock medium must have been an extremely meager source of nitrogen or other essential organic matter. This lack of available nitrogen may account for slower division-rates in cultures started with one or two individuals in relatively large volumes when compared with those given with larger numbers in the inoculum.

The present results concur with those of Dusi (1937), Hutner (1936), Jahn (1935a), Hall and Schoenborn (1939), and others, in that growth was not as abundant or as assured in an inorganic medium as in infusions of several types. Cessation, and finally death, was a common result from cultures in salt solutions in large volumes. This inability of cultures to continue growth regularly in sterile inorganic salt media or to attain maximal numbers in mass cultures in such media, even with bacteria present, undoubtedly is reflected in the early losses in isolation cultures.

Stock cultures grew abundantly in wheat infusions which were made by boiling 10 grains of dried wheat in 100 cc. of distilled water for 10 minutes and allowing it to stand 24 hours before being inoculated with several drops from an established culture. Ten thousand *Euglena* per pipette drop (0.05 cc.)² was a typical count in such infusions.

The original stock culture was developed from an isolated individual which produced twelve euglenae in 24 hours. The division-rates of subclones were carefully studied to detect possible variation in cultures with small volumes. In wheat infusions, where bacterial contamination and evaporation are great, no variation in division-rates, which

² Unless specifically stated to the contrary, the drops used in this study contained approximately 0.05 cc.

were statistically significant, appeared in 26 hours. In 36, 42, and 66 hours significant variations did occur. Readings at the end of 24 hours only are used in this paper; hence no significant variation is to be expected as a result of differences inherent in the euglenae themselves.

In preparation of any series for an experiment the following dilution methods were employed to reduce the number of bacteria. This method was used for all experiments unless otherwise designated as sterilized by exposure to ultra-violet irradiation, as described at the beginning of Section IV.

One drop of a stock culture, approximately 2 weeks old, was mixed with 5 cc. of autoclaved salt solution in a sterile watch glass³ and was allowed to stand 1 hour. This is called "dilution Bath A": it diluted the stock 101 times. Bath B was made by transferring 1 drop of Bath A to 10 drops of sterile salt solution, diluting the stock 1,111 times. Euglenae were kept in this bath 10 minutes. Bath C involved a transfer of 5 micropipette drops (0.0025 cc.), each microdrop containing one *Euglena*, to 5 large drops (0.05 cc.); and this mixture was allowed to stand 20 minutes. This last bath brought the total dilution of the stock medium to 23,331, and the concentration of the stock became approximately 0.00004.

As euglenae were transferred from Bath C to sterile salt-solution cultures of from 1 to 40 drops, the cases of greatest concentration of the stock present in any of the experiments would occur with eight euglenae present in 1 drop. This further dilution would contain approximately 0.000002 concentration of the stock materials of bacteria.

These changes in concentration were gradual enough to avoid shock, since there was no readily apparent change in motility or morphology.

Bacterial counts (Table 1), employing plating methods as used in standard water analyses, were taken at various intervals in the dilution series. Counts made at the end of the first 8 minutes in Bath C showed fewer bacteria in the salt solution than at the end of 10 or 12 minutes, after which time there was no increase. Tests made at the end of 15 minutes in Bath C, according to standard bacteriological methods, rarely showed more than seven viable bacteria present per cubic centimeter. Later in the present paper, when cultures are said to be "sterile" or "bacteria-free," this degree of sterility is meant.

Stock cultures tended to become alkaline, pH 8.1-8.5, whereas the sterile salt solution was buffered to a pH of 7.6. Subcultures in small drops kept the same pH at least 24 hours. The change from stock-culture media of greater alkalinity apparently offered no great physiological hazard, although this may have been one of the reasons that reproductive rates were greatest in wheat solutions.

Because of the use of carbon dioxide by autotrophs, carbonic acid does not accumulate. If CO₂ utilization is a measure of division-rate or of metabolism, the more alkaline media might indicate more complete metabolism; and, likewise, greater acidity might be associated with less complete use of materials in the medium and with comparatively slower division-rates.

Quantitative studies on the optimal pH for the growth of *E. gracilis* in various types of organic media and with various temperature and light relationships have been reported by Kostir (1921), Jahn (1931, 1932, 1935a), Hall (1933), Wang (1928), and others.

Measurement of CO₂ consumption, O₂ production, and oxidation-reduction potentials

³ All glassware was washed with soap and water and rinsed with flowing hot tap water or was allowed to stand in glass-cleaning solution overnight and rinsed thoroughly with running hot tap water and dried with clean linen towels, wrapped in heavy tissue paper and sterilized in a dry oven at 190° C.

for *Euglena* species have not been reported. Jahn (1933) found, with *Chilomonas*, that the reduction of sulphur was very important in establishing high division-rates. Certainly, CO₂ sources are of great importance to a chlorophyll-energy system. Because

TABLE 1
NUMBERS OF COLONIES OF BACTERIA IN 1 CC OF VARIOUS SALT-SOLUTION
BATHS AND CULTURES OF *Euglena gracilis* EMPLOYING THE
AGAR-PLATE COUNTING METHOD
(Average of ten cases, 1935 and 1936)

Section	Culture	Time Elapsed	Mean	Standard Deviation
A*	Stock	2 wk.	4,330,000 0	500,400 0
	Bath A	60 min.	34,200 0	8,600 0
	Bath B	10 min.	4,600 0	1,200 0
	Bath B	20 min.	8,300 0	2,300 0
	Bath C	8 min.	2 0	1 0
	Bath C	10 min.	3 4	1 0
	Bath C	12 min.	3 3	1 0
	Bath C	15 min.	3 0	0 1
B†	a) Washed sterile cultures in sterile salt solution (8 euglenae present)	10 min.	1 00	0 05
		24 hr.	0 75	0 01
	b) Ultra-violet radiated 2-minute salt-solution culture (8 euglenae present)	10 min.	1 20	0 04
		24 hr.	0 06	0 00
	c) Ultra-violet radiated 3-minute salt-solution culture	10 min.	0 00	0 00
		24 hr.	0 01	0 02
C‡	a) X concentration No euglenae present	10 min.	3,100,700,100	75,000
		24 hr.	3,107,900,100	49,100
	8 euglenae present	10 min.	3,100,600,060	54,200
		24 hr.	3,111,637,940	71,230
	b) 0.001 X concentration No euglenae present	10 min.	2,876,000	4,920
		24 hr.	2,500,900	5,700
	8 euglenae present	10 min.	2,914,912	7,200
		24 hr.	3,241,617	7,140

* Bacterial counts from dilution baths in preparation for washed-sterile euglenae cultures.

† Bacterial counts from 1 drop of washed-sterile, irradiated 2-minute, and irradiated 3-minute cultures with eight euglenae present at the beginning and end of an experiment.

‡ Bacterial counts from *Pseudomonas* suspensions of X and 0.001 X concentrations with and without euglenae present.

division-rate is notably higher in organic media, it is easily surmised that a major function of certain bacteria or of organic radicals may be that of serving as links in the carbon or nitrogen cycles of *Euglena* metabolism.

Temperature has been constant to within 0.5° C. in any one series of experiments.

Temperatures of 23°, 25°, and 27° C. have yielded approximately similar results in 1935, 1934, and 1936, respectively; at least, no significant differences could be found in the average division-rates shown by comparable cultures in these three series.

Constancy of light, humidity, and temperature were maintained by use of a specially constructed chamber, which was operated in a room with controlled temperature and humidity. This chamber, designed by Dr. W. C. Allee, is a cylinder 3 feet in diameter and 3 feet in height with a revolving press-board floor. It had as its source of light six 100-watt "daylight" mazda bulbs, each with a reflector and suspended from an overhanging disk 3 feet above the floor of the chamber. The light passed through a heat screen of over 2 inches of filtered-water plate glass and caused little distortion of the light. During an experiment the chamber was lighted constantly, day and night. The thermograph records of the temperature within the chamber showed the variation to be no greater than 0.5° C. The humidity of the constant-temperature room was under positive control and remained around 67 ± 2 per cent; within the light chamber there was less variation from this mean. It was also possible to place the crystallization dishes holding the depression slides of an experiment in this chamber without any movement of the cultures within a given experimental period.

III. A COMPARISON OF NUMBERS PRODUCED IN CULTURES INOCULATED WITH ONE TWO, FOUR, AND EIGHT INDIVIDUALS IN VARIOUS SMALL VOLUMES

Cultures of *Euglena* (washed free from bacteria, as described in the previous section) showed certain similarities in division-rates dependent on the number of organisms which were placed in a given volume. As always in these experiments, all euglenae were from the same clone. In these particular tests they were cultured in from 1 to 40 drops of fresh sterile medium. Series were arranged so that there were eight individuals isolated singly in eight different depression slides; four slides were inoculated with two organisms each; two slides similarly were seeded with four and two others with eight individuals each. One of the last served as a control on which bacteriological tests were made.

This set of sixteen slides constituted one experimental series. Forty individuals were required to inoculate such a series. Each organism was equally and separately washed and was finally transferred to its respective slide within a half-hour of other members of the series. Each slide was covered with a thin cover slip, which was held in place by a film of vaseline or sterile water. Evaporation was reduced to a minimum by placing the covered slides in a moist culture chamber composed of a large crystallization dish in the bottom of which was about an inch of water; the slides were placed on a white platform above the water. Such a chamber, holding eight depression slides, was transferred to the constant light, temperature, and humidity chamber already described. The two crystallization dishes which housed a single experimental series were kept close together, so that all slides received approximately equal illumination.

The subcultures were left undisturbed until they were removed to be counted at the end of 24 hours. The control slide was tested bacteriologically by the plating method. Contamination could not be determined until another day had elapsed; hence, no experiment was concluded until at least 52 hours after it began. Twenty-four-hour counts of *Euglena* proved most satisfactory; in 48 hours there was evidence of slight evaporation, even though the slides had not been inspected in the meantime.

Depression slides were used for volumes of from 1 to 5 drops; watch glasses were used

for those of 10, 20, and 40 drops. The latter were covered with heavy glass slides made to fit and sealed with vaseline. Methods of sterilizing all glassware were described in Section II of this paper.

The data accumulated in three years of experimentation have been summarized in Figure 1 since there were no significant differences in the results for any one year which could make invalid a treatment of the data as a whole. The graphs plot the average total

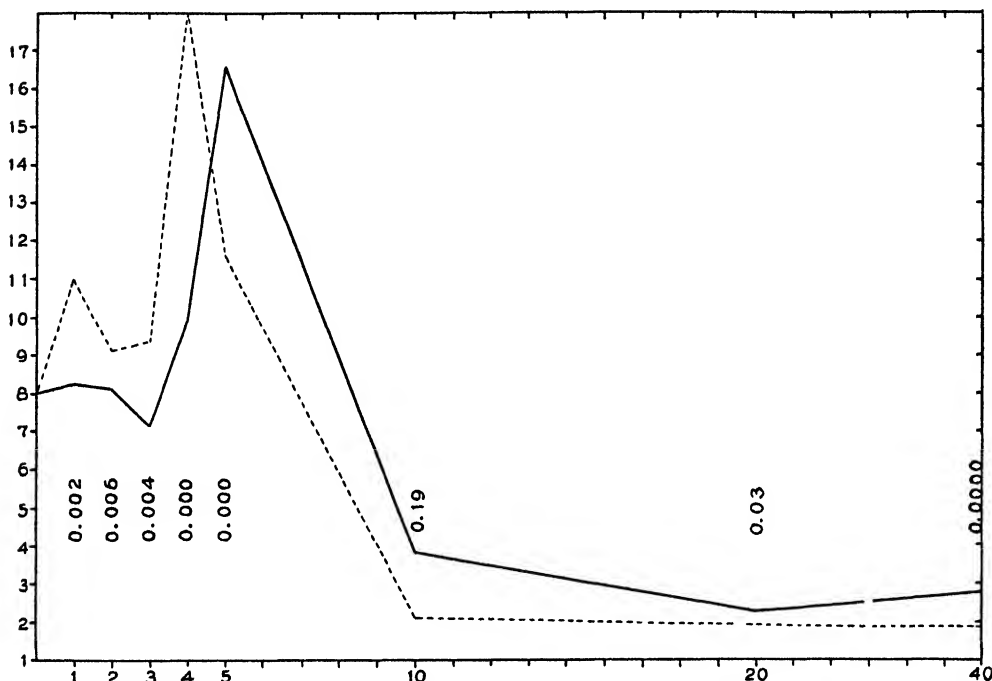


FIG. 1.—Average total numbers of *Euglena gracilis* present after 24 hours in cultures inoculated with one and two (dotted line) contrasted with four and eight (solid line) individuals with statistical probability of the differences. Ordinates, average total numbers present after 24 hours; abscissas, volumes in drops. Numerals give the statistical probability (P) of the observed differences at the several points. This was determined by Student's method for paired experiments. $P = 0.03$ means that there are three chances in one hundred of finding as great differences by random sampling. $P = 0.05$ is usually considered to be the extreme upper limit of statistical significance. The smaller the value of P , the greater the significance.

number of individuals produced in 24 hours in cultures started with one and two euglenae (broken line) and those started with four and eight (solid line) in the various volumes. In addition, the statistical probability of the differences are given for each volume.

Table 2 shows the number of series which have been summarized in Figure 1. The fully expanded table, showing comparisons for the different volumes for the experiments conducted in 1934, 1935, and 1936, summarized by years, is given in detail elsewhere (Sweet, 1937). The summary in Table 2, together with the statistical probabilities shown on the figure itself, gives some indication of the reality of the recorded differences. The

fact that essentially similar results were obtained in three different periods of experimentation adds to their reliability.

In the smaller volumes the cultures started with fewer euglenae reproduce faster than those seeded with four or eight individuals. The mean difference in the average total numbers in the cultures inoculated with one and two euglenae is greater than in those having four and eight in the inocula in 1, 2, 3, and 4 drops; the actual difference is 2.92, with $P = 0.00001$. In 4 drops taken alone, the same cultures also reproduce more rapidly; the difference is 9.5 with $P = 0.0000$.

In 5 drops conditions are reversed; the difference is 6.26 with $P = 0.0000$. In the largest volumes tested, 20 and 40 drops, the average total numbers maintained by the two groups show statistically significant advantage for the larger number in the inocula, with a difference of 1.00 and $P = 0.0000$.

There is very little difference, none of statistical significance, in the average division-

TABLE 2
A SUMMARY SHOWING THE AMOUNT OF EXPERIMENTATION
USED AS A BASIS FOR FIGURE 1

CULTURE VOLUME (IN DROPS)	NUMBER OF SERIES		
	1934	1935	1936
1.....	16	16	9
2.....	16	15
3.....	13	13	13
4.....	10	3	13
5.....	8	12
10.....	8
20.....	11	7
40.....	15	9

rates of each group in the three smallest volumes of 1, 2, and 3 drops, in spite of the fact that the volumes are doubled and tripled. There is, for example, no significant advantage or difference in the numbers produced in cultures started with a single individual in 1 drop, as compared with 2 or with 3 drops of culture medium.

As seen in Figure 1, decided differences in division-rate appear at the transition from 3- to 4-drop cultures; the average rates are doubled, or practically so, for the cultures inoculated with one and two organisms; and there is a 50 per cent acceleration for those started with four and eight individuals. Cultures with one and two euglenae in the inocula attain the highest rates of reproduction to appear anywhere in the series tested. The acceleration in cultures with least numbers in the inoculum, as compared with those with greater, a phenomenon which is typical of the smaller volumes, is greatly enhanced in 4 drops. This volume of 4 drops represents the optimal volume for seedings of one and two euglenae.

In 5 drops the cultures inoculated with the greater numbers show significantly higher reproductive rates, and these reach their maximum in 5 drops. Cultures seeded with one and two individuals decline in division-rates from cultures of 4 drops to those of the 5 drops almost as rapidly as they increased from the smallest volumes to those of 4 drops.

In 4 drops the cultures seeded with eight euglenae had least numbers at the end of 24 hours; in 5 drops these cultures increased the most and attained a maximal number for all volumes tested of those seeded with eight organisms. The optimal volume of cultures inoculated with four and eight individuals is, under the conditions of these experiments, 5 drops; whereas, cultures seeded with one and two individuals reached their maximal numbers in the 4 drops.

In the twenty series tested in 5 drops the cultures inoculated with four and eight individuals gave an average total number of 16.88 at the end of the first 24 hours; similar cultures started with one and two euglenae produced an average of 10.62. The chance of this occurring in random sampling is less than once in several thousand times, $P = 0.0000$.

When identical volume relations obtain per individual in the inoculum and the numbers produced are compared, the cultures started in 5 drops show significantly higher rates than do those of either supraoptimal or suboptimal volumes. One comparison was possible with the suboptimal volumes, and six with the supraoptimal. There were seven cases in which cultures of 4 drops could be contrasted with suboptimal volumes, in which identical volume-per-individual relations existed; and of these, five gave significantly more euglenae in the larger volume. One of the two cases in which the cultures in 4 drops could be compared with supraoptimal volumes showed significantly more organisms produced in 4 drops.

In brief, from this type of comparison it is clear that it is not only the relationship of volume per individual which acts to account for differences in division-rates, but there is, under the conditions of these experiments, a volume which tends to be optimal because it is conducive to greater division-rates almost regardless of the number of inoculated euglenae or initial volume-per-capita relationships.

In 10, 20, and 40 drops (Fig. 1), which constitute the three largest volumes tested, the division-rates fall for all cultures, irrespective of size of inoculum; maintenance of original numbers is impossible; and the cultures seeded with four and eight individuals show significantly greater ability to survive.

In 10 drops considered alone, the cultures with eight initial individuals show significantly greater numbers than do any other cultures; the probability of these differences⁴ is in the cases of 1/8 $P = 0.004$, 2/8 $P = 0.0000$, and 4/8 $P = 0.004$. The cultures with four and eight individuals in the inocula, when grouped as in Figure 1, however, do not show significant advantage over those with one and two euglenae seeding a culture in this supraoptimal volume.

In 20 drops there is a slightly significant difference in the division-survival rates of the cultures; four and eight organisms in the inocula make the better showing ($P = 0.03$). In 40 drops cultures with four and eight in the inocula, in 24 parallel cases, show a very reduced but a significantly greater number present after 24 hours than do cultures started with but one or two euglenae.

With the suboptimal volumes one can postulate that one or more of the following factors are acting: (1) too great concentration of excretory products; (2) too great concentration of a growth-promoting substance or substances; (3) too great evaporation and hence a too rapid or too great change in salt concentration or too concentrated an

⁴ "1/8" refers to a contrast between the division-rate of the cultures inoculated with one organism and those inoculated with eight. The statistical significance of the difference is given as P .

end-product; (4) too great an intake or accumulation of oxygen and far too rapid a loss of carbon dioxide; and (5) too few bacteria or bacterial end-products.

With the supraoptimal volumes the converse of these same conditions holds as possible responsible agents to account for the harmful effects of the greater volumes: (1) too much toxic material per organism; (2) too great dilution of growth-promoting substance or substances; (3) too slow loss of carbon dioxide; (4) too slow intake of oxygen; (5) too slow evaporation, i.e., too slow a change in osmotic pressure; and (6) too many bacteria or bacterial end-products.

In view of the instances in which equal volume-per-organism relations have shown that volume per individual is not, by itself, controlling the division-rates (as explained on p. 182), it is likely that those factors connected with surface-volume differences are those most active in altering the numbers produced or surviving. Points (3) and (4) of both foregoing lists and (5) of the second list are at once subject to surface-volume differences. It is easy to see that in the suboptimal volumes evaporation may play an important role and that, by change in the osmotic pressure of the media, may be deterring division. This is probably the major cause of depression in suboptimal volumes.

On the supraoptimal side of the curve the causal relations are less clear. At present we can only suggest that the relatively increased mass and relatively decreased surface may be associated with the depression, or that the medium is toxic and the toxicity shows up most readily when there is a large volume per individual. This well-established mass effect is discussed at some length by Allee (1931, 1938). As might be expected from similar studies, the toxic effect was less pronounced in cultures seeded with the greater number of euglenae, notably in 10, 20, and 40 drops. Although the average population for such cultures always decreased, differential survival was exhibited in favor of increased numbers in the inocula.

The greatest numbers produced in the small-volume, suboptimal cultures, when contrasted to the numbers maintained in the supraoptimal cultures, is evidence that the conditions obtaining in these small volumes approach the optimal more nearly than do those in the larger volumes for the size of inocula used.

The phenomenon which appears most dramatic in this survey of division-rates of washed-sterile euglenae is the rapid reversal of the size of the inocula which yields maximal numbers. Four and 5 drops represent the optimal volumes for division-rates of euglenae in this type of culture. With the addition of 1 drop of culture media the optimal number of euglenae seeding cultures was changed from the smaller to the greater number in the inocula. It is not clear why this reversal should occur so rapidly and in these particular volumes. However, such a reversal is in line with the experience of certain workers with similar relationships for ciliates, notably Peterson (1929) and Johnson (1933, 1936), except that with their work the transition was less sudden.

Evidence of the Robertson effect or of some modification of it is present in the cultures in 5 drops of these salt-solution series which are relatively free from bacteria. Reproduction and/or survival rates "are accelerated by the presence of contiguous organisms in a limited volume." In the supraoptimal volumes, of 10, 20, and 40 drops, there is certainly a loss of individuals and probably a diminished division-rate in the survivors; even so, more individuals are present in cultures inoculated with greater numbers.

There is no evidence that this effect, even in 5 drops, is to be accounted for by "allelotaxis" as explained by Robertson. The contribution of nitrogen to the media, made possible by the death of one or more individuals in the larger volumes inoculated

with greater numbers, might account for the observed effect. Also, because of the fact that volume per organism is not in all cases a controlling factor, there is evidence that surface-volume factors may be active. More analyses are necessary before the explanation of the observed differences can be established.

IV. EFFECTS OF NUMBERS PRESENT ON DIVISION-RATES OF *Euglena gracilis* FREED FROM BACTERIA BY ULTRA-VIOLET IRRADIATION

Euglenae washed, as before, through baths A and B were irradiated⁵ 2 and 3 minutes in these experiments and were then transferred by micropipette to sterile salt-solution cultures (as explained fully in Sec. II). Bacterial counts taken 10 minutes after transfer showed less than two colonies per 0.05 cc. by plate-counting methods (Table 1, B), and after 24 hours in culture even fewer bacteria were present. The method was, therefore, not any less effective in obtaining practically sterile cultures than the dilution method and involved one less transfer.

The results presented on the effects of irradiation on division-rates are those occurring because of the reactions of 2- and 3-minute irradiated organisms in sterile experimental environments when volumes and numbers of seeded *euglenae* are the only known variables. Every individual used in these experiments was irradiated in the same volume and with approximately one hundred other *euglenae* present during the exposure to ultra-violet.

The effects of irradiation for 2 and 3 minutes on populations of various sizes in 1 and 4 drops are shown in Figure 2. The numbers present in these series after 24 hours represent an ability to survive as well as to reproduce, and clearly reveal in these capacities differences which are dependent on the size of the initial population.

It is apparent at the outset that such comparisons as are shown in Figure 2 give evidence that growth of *euglenae* is not as great following irradiation as after treatment in dilution baths. Hence, we are dealing with an effect which is more harmful than that of washing.

The direction of increase in numbers produced per size of inocula is not the same in the irradiated as in the washed-sterile series, except with 2-minute irradiated *euglenae* in 4 drops where conditions are least toxic of any of the irradiated series. In 4 drops, washed-sterile *euglenae* divided significantly more rapidly than did 2-minute irradiated *euglenae*; in the cultures started with two individuals the value of *P* for the difference in rate was 0.0000; with four individuals in the inocula, 0.0000; with eight, 0.001; and with one and two considered together, 0.000.

The most harmful effects occurring in these series of irradiated *euglenae* are in those grown in 1 drop after 3 minutes of irradiation. Three-minute irradiation is decidedly more deleterious than that for 2 minutes, when the *euglenae* are cultured in either of the two volumes studied. In fact, in cultures in 1 drop and with irradiation for 3 minutes (Fig. 2, c) there is a population decrease in 24 hours in the cultures seeded with one, two, and four *euglenae*; and survival only is measurable. An increase in numbers present occurs only in the cultures with the largest number (eight) in the inocula. In this series an increase in density of initial population is, in every instance, of advantage: statistical

⁵ A Cooper-Hewitt quartz mercury-vapor lamp was used; it ran at 110 volts D C. and about 4 amperes, and at 66 cm. distance from the center of the arc to the culture being irradiated. It was over 10 years old and had been much used.

significance is attached to each difference in the numbers surviving or produced in cultures with successively greater numbers in the inocula. Table 3 gives the results of a comparison of the differences.

A significant advantage is also accorded larger-sized inocula in the group of experiments of 1 drop with euglenae irradiated for 2 minutes. The value for P for differences

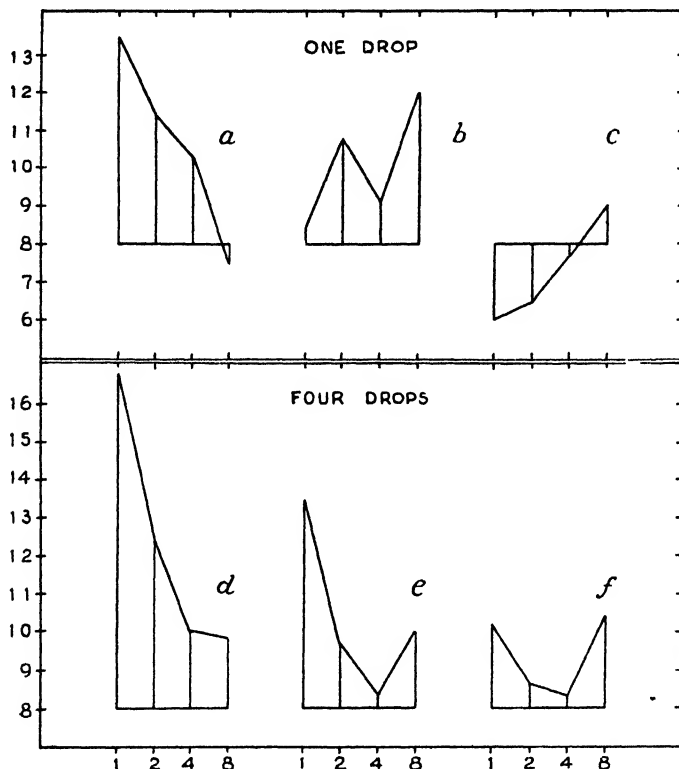


FIG. 2.—Differences in numbers of *Euglena gracilis* present after 24 hours in cultures inoculated with washed-sterile or 2- and 3-minute irradiated euglenae in 1 and 4 drops with varying numbers in the inocula. Ordinates, average total numbers present after 24 hours; abscissas, numbers in the inocula. *One drop*: (a) washed-sterile euglenae; (b) 2-minute irradiated; (c) 3-minute irradiated. *Four drops*: (d) washed-sterile; (e) 2-minute irradiated; (f) 3-minute irradiated.

between cultures started with two, as compared with one, individual is 0.04; for 4/1 is 0.0000; and for (4 and 8)/(1 and 2) P is 0.013.

In the larger volume, 4 drops, all cultures are capable of increasing their numbers; and the larger inocula are not significantly more successful than they were in the smaller volumes. Rather, the least numbers in the inocula have a relatively accelerated reproductive rate when only 2-minute irradiation is used, and the differences in the division-rates between smaller- and larger-sized inocula are significant (Table 4).

With euglenae irradiated for 3 minutes and cultured in 4 drops no difference is significant for any size of inocula, and the smaller inocula are not significantly retarded in

this volume. The larger volume is less deterring, therefore, to division of euglenae irradiated 2 or 3 minutes; and the final conditions approach those found in similar cultures started with washed-sterile individuals.

The fact that more harmful effects are seen in the 1-drop series, with either 2- or 3-minute irradiation, than in 4 drops, is in accord with the findings with the washed-sterile series in which optimal culture conditions seemed to prevail in 4 and 5 drops. However, in comparison of population densities in these two volumes, there are effects

TABLE 3
COMPARISON OF AVERAGE NUMBERS PRESENT WITH VARIOUS
SIZED INOCULA OF EUGLENAE IRRADIATED FOR
3 MINUTES AND CULTURED IN 1 DROP

Comparison of Inocula Size	Average Total Numbers Present	Statistical Probability
2/1	6.5/6.0	0.000
4/1	7.6/6.0	0.02
8/1	9.0/6.0	0.0000
4/2	7.6/6.5	0.0051
8/2	9.0/6.5	0.0000
8/4	9.0/7.6	0.000
(1 and 2)/(4 and 8) ..	12.5/16.6	0.0000

TABLE 4
COMPARISON OF AVERAGE NUMBERS PRODUCED WITH VARIOUS
SIZED INOCULA OF EUGLENAE IRRADIATED FOR
2 MINUTES AND CULTURED IN 4 DROPS

Comparison of Inocula Size	Average Total Numbers Produced	Statistical Probability
1/2	13.5/9.7	0.0098
1/4	13.5/8.3	0.0004
1/8	13.5/10.0	0.0092
2/4	9.7/8.3	0.0000
8/2	9.7/10.0	0.0098
8/4	9.7/8.3
(1 and 2)/(4 and 8) ..	23.2/18.3	0.0032

on division-rate or on survival which indicate that some factors may be operating which are also dependent on the volume-per-individual phenomena.

In brief, under the conditions of these particular experiments, toxicity or deleterious factors in general, as manifested by lessened division-rates, are most significant in small volumes and when least numbers compose the inocula. As volume increases and/or numbers in the inocula increase, such factors show diminished effects.

V. THE EFFECTS OF NUMBERS INOCULATED ON RATE OF DIVISION IN CULTURES WITH SEVERAL SPECIES OF BACTERIA OF KNOWN CONCENTRATION

The following series of experiments were arranged in parallel fashion in order to ascertain the effects of symbionts and so compare the reproductive rates of *E. gracilis* in

the presence of mixed bacteria in known concentration with those given in bacteria-free inorganic media.

The media for these experiments were prepared in the following manner. Stock cultures were made in 100 cc. of the usual salt solution in small Erlenmeyer flasks. These were seeded with one or more pipettefuls of euglenae from the standard wheat-infusion stock and were well contaminated with bacteria of the wheat cultures. One cubic centimeter of supernatant fluid obtained by centrifuging 10 cc. of these salt-solution stock cultures was diluted with ten parts of sterile salt solution. Five species of bacteria were present in approximately the same proportion as found in the stock wheat infusions. The concentration of bacteria, as checked by colony-counting methods, was about 3,000,000 per cubic centimeter (Table 1, C). This approached the equivalent of a 0.001 X concentration suspension as used by Johnson (1933, 1936) and described in the next

TABLE 5
COMPARISON OF AVERAGE TOTAL NUMBERS PRODUCED IN NONSTERILE
CULTURES INOCULATED WITH VARYING NUMBERS OF
Euglena gracilis IN 1 DROP
(Average of twelve series)

Inoculated Number	Average Total Number	Comparison	Probability
1.....	6 3	1/2	0.14
2.....	8 75	1/4	0.0000
4.....	11.7	1/8	0.0000
8.....	10 75	2/4	0.08
1 and 2.....	7 75	2/8	0.048
4 and 8.....	11 2	4/8	0.18
		(1 and 2)/(4 and 8)	0.0000

section. These cultures will be designated as "nonsterile cultures," to avoid confusion with other bacterial suspensions referred to in Section VI.

In 1-drop cultures of the nonsterile series (see Table 5) euglenae divided more rapidly when seeded with two, four, or eight organisms than if but one was introduced. The results were similar to those obtained with 1-drop cultures of euglenae which had been irradiated for 2 minutes, and were opposite to those found using washed-sterile euglenae in the same volumes.

The cultures started with single individuals are most markedly altered in their division-rates in all these series: they show striking acceleration in the washed-sterile inocula and lack of maintenance of numbers in the nonsterile cultures and only a slight increase in the cultures inoculated with irradiated euglenae. Are the factors which accelerate division in small-volume cultures with smallest inocula of washed-sterile individuals absent in these nonsterile populations, or are they overborne by other factors?

Whatever the causal mechanism or mechanisms may be, the effect appears to be related to the inability of the smaller inocula, more especially of isolated euglenae, to flourish under the conditions that obtain in this so-called "nonsterile" series.

VI. COMPARISONS OF NUMBERS OF *Euglena gracilis* PRESENT AFTER 24 HOURS IN SUSPENSIONS OF CONTROLLED CONCENTRATIONS AND SPECIES OF BACTERIA

Each of the five species of bacteria which composed the natural flora of stock cultures of *E. gracilis* (either wheat or salt-solution stocks) was isolated on nutrient agar plates,

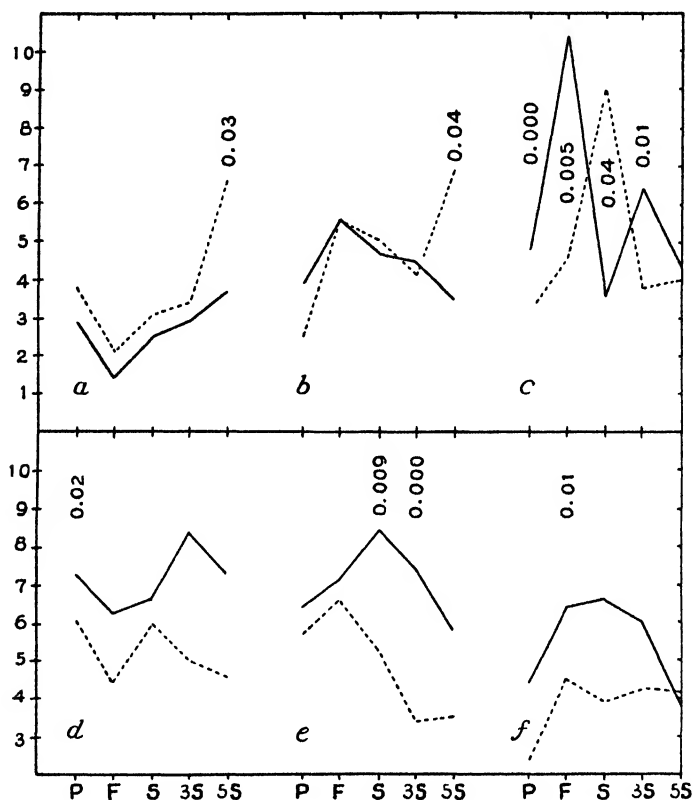


FIG. 3.—Differences in numbers of *Euglena gracilis* present after 24 hours in six concentrations of various bacterial suspensions in cultures (1 drop) inoculated with one and two (dotted lines) compared with four and eight (solid lines) washed-sterile euglenae. Ordinates, average total numbers present; abscissas, types of suspension. P, *Pseudomonas*; F, *Flavobacterium*; S, *Staphylococcus*; 3S, three-species suspensions; 5S, five-species suspensions. When significant, the statistical probabilities of the differences are given (see legend to Fig. 1). The various figures show different concentrations of bacteria as follows: a, X; b, 0.1 X; c, 0.01 X; d, 0.001 X; e, 0.0001 X; f, 0.00001 X.

and a platinum loopful of a 24-hour colony of each species was transferred to 10 cc. of nutrient broth and incubated 24 hours. Then a loopful was transferred from the nutrient broth suspension to 10 cc. of sterile Osterhout salt solution to compose an X concentration of a known species of bacteria. Appropriate dilution gave a 0.1 X, a 0.01 X suspension, etc.

The bacteria used were *Flavobacterium*, a pigment-producing cocci, *Staphylococcus*,

sp., and *Pseudomonas fluorescens*. The species of the last genus was definitely established. Two gram-negative, non-spore-forming rods were also in stock cultures and were used in the cultures of the five mixed species. The three species suspensions were made up of *Flavobacterium*, *Staphylococcus*, and *Pseudomonas*. For the mixed cultures, of three and five species, loopfuls from three or five of the nutrient broth suspensions were placed in 30 and 50 cc., respectively, of sterile salt solution to compose X concentrations; and appropriate dilutions were made as indicated. *Euglenae*, in preparation for growth in

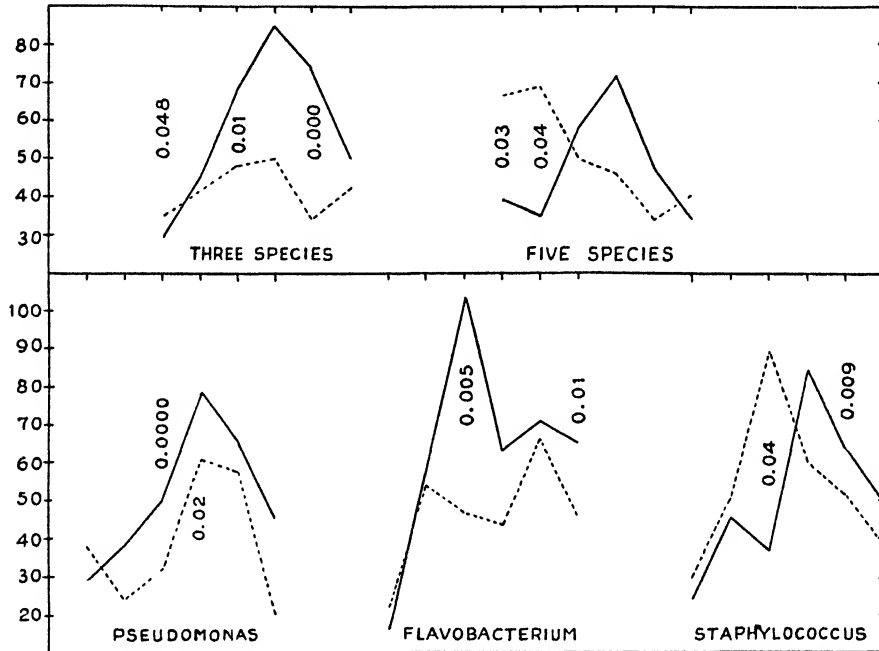


FIG. 4.—Differences in numbers of *Euglena gracilis* present after 24 hours in five bacterial suspensions (1 drop) of varying concentrations with one and two individuals in the inocula (dotted lines) compared with four and eight (solid lines). Ordinates, average numbers present; abscissas, concentrations of the suspensions for each pair of graphs; the points show the following concentrations of bacteria, X, or X, 0.01 X, 0.001 X, 0.0001 X, and 0.00001 X. When significant, the statistical probabilities of the differences are given.

these media, were passed through the three dilution baths and were mouth-pipetted to the final culture-slide, as in all the washed-sterile series. All cultures were made in 1 drop of the standard salt solution.

Figures 3 and 4 schematize the cumbersome details accumulated in this series in a relatively simple fashion. The data from which these graphs are taken, together with the statistical calculations, are available, as are all the data for tables for previous sections (Sweet, 1937). Results from the cultures inoculated with one and two individuals have been considered together (dotted line), and, likewise, those inoculated with four and eight individuals (solid line).

Both figures present the same material but with different emphases. Figure 3 shows

the six concentrations separately, with the numbers of *Euglena* present after 24 hours in the five different types of bacterial suspension. Figure 4 presents the five different types of suspensions separately, with the numbers present in the various concentrations of each type of suspension. The two figures can be cross-read. The following summary of the material here presented uses both graphs.

1. There is a lack of maintenance of the numbers of *E. gracilis* inoculating a series in the majority of cases in these bacterial suspensions of different species and concentrations. In the sixty cases, only four instances occur in which an average of more than eight (the original inoculated for each series) individuals are present in these suspensions after 24 hours. Hence, for the most part we are measuring survival, not maintenance or growth of cultures.

2. From glancing at Figures 3 and 4 it is readily observed that in most cases the cultures inoculated with the greater numbers (four and eight individuals, heavy line) are capable of sustaining greater numbers than are those with fewer numbers in the inocula (one and two, dotted line).

3. This dominance of the larger-sized inocula occurs in nearly every instance in the less concentrated suspensions. (Refer to Figure 3, *d*, *e*, and *f*, the 0.001 X, 0.0001 X, and 0.00001 X concentrations.) This is true regardless of the specific type of suspension. However, this increase in numbers is significant in only four of the fourteen individual instances (Fig. 3, *d*, *Pseudomonas*; *e*, *Staphylococcus* and three species; and *f*, *Flavobacterium*).

4. In the more concentrated suspensions there is less uniformity in the results. In the 0.01 X concentration, three cases of significantly greater numbers occur in favor of the larger inoculating group, in the *Pseudomonas*, *Flavobacterium*, and three-species suspensions. The smaller inoculating group grow best in the *Staphylococcus* suspension at this concentration. Also in the 0.1 X concentration, the most significant difference occurs in favor of the smaller inoculating group in the five-species suspensions (Fig. 3, *b*).

5. In the most concentrated suspensions, X, the smaller inoculating group is consistently more numerous, but significantly so only in the five-species suspensions (Fig. 3, *a*).

6. In the most dilute and most concentrated suspensions—the X, 0.1 X, and 0.00001 X—numbers present in 24 hours are reduced more than in the moderately concentrated suspensions (Fig. 3, *a*, *b*, and *f*; cf. *c*, *d*, and *e*).

7. Hence, it is apparent that, where there are significant differences in the more concentrated suspensions, the smaller the numbers in the inocula the greater the chance of surviving; and in the less concentrated suspension, the larger the number in the inocula the greater the chance of survival. The transition occurs at the 0.01 X concentration.

8. In viewing these same relations in Figure 4, in order to note the specific effects of varying bacterial species in suspension and in a range of concentrations, no one type of suspension is much different in effect than any other. In checking the average total numbers in each suspension, *Pseudomonas* is least conducive to survival, with an average of 4.55 living after 24 hours from an initial seeding of 8.0; the five-species suspension next, 5.01; the three-species, 5.11; *Flavobacterium*, 5.33; and *Staphylococcus*, sp., the most conducive, 5.41. These differences are, obviously, not great.

9. Significant differences in survival in each of the suspensions are not common.

a) In the *Pseudomonas* suspensions, statistically significant differences in numbers

present occur in the 0.01 and the 0.0001 X concentrations in favor of larger numbers in the inocula, although in all the concentrations of this type of suspension, except in the most concentrated, the larger number in the inocula are in advance of those with smaller numbers.

b) In the *Flavobacterium* suspension, again larger numbers do best in the concentrated suspension and are significantly more successful in sustaining *Euglena* in the 0.01 and the 0.0001 X; in fact, in the 0.01 concentration, increase in numbers occurs beyond the maintenance level.

c) In the *Staphylococcus* suspensions, only one significant difference occurs, and this in favor of the cultures with one and two individuals in the inocula in the 0.01 X concentration. In this group smaller numbers in the inocula are more numerous in the three most concentrated suspensions. In the more dilute suspensions the greater numbers are in advance—significantly so in one case, the 0.0001 X concentration.

d) In the three-species suspensions, three significant differences occur in favor of larger-sized inocula, in the 0.1 X, 0.01 X, and 0.0001 X concentrations. Only in the most concentrated suspensions do the smaller-sized inocula succeed in sustaining *Euglena* better than do larger-sized inocula; but the numbers are very reduced, and the difference is not significant.

e) And lastly, in the five-species suspension the significantly greater numbers occur in the X and the 0.1 X concentrations with smaller-sized inocula; and in the less concentrated, 0.0001 X concentration, with the greater-sized inocula.

Hence, in the eleven cases of statistically significant differences in the numbers surviving in 24 hours in such bacterial suspensions, seven of them occur in cultures with greater numbers in the inocula, three instances in the 0.01 X, one in the 0.001 X, two in 0.0001 X, and one in the 0.00001 X concentrations. The three cases in which smaller numbers in the inocula favor the survival of euglenae were in the more concentrated suspensions, X, 0.1 X, and 0.01 X. It can be said that, in general, in each of these specifically different bacterial suspensions significant differences in survival are apparently related to the numbers seeded and are somewhat more assured in cultures with greater numbers in the inocula in the moderately dilute concentrations.

The division-rates in these bacterial suspensions, when viewed as a whole, are not as great as in any other of the series studied—washed-sterile, irradiated, or even non-sterile. In fact, usually maintenance is not possible: only survival-rates can be compared. There appears to be some deterrent of division present in practically all the concentrations, species, and combined species tested. However, because rates are not lower in at least one concentration of the five types of suspension, generally the 0.01 X or 0.001 X, than in some of the media already studied, there may be no factor except concentration of the specific bacterial suspension which is acting to lower rates.

In the five-species suspension, where the same species are present as in the stock and nonsterile cultures, it would be expected, perhaps, that in equal quantities the same or similar rates of division might obtain as in suspensions with similar proportions of the stock cultures. But numbers of bacteria present are considerably less. It appears that in equal quantities there may be some one or more species present in greater or less quantity than is conducive to growth of the culture in this short period. The *Pseudomonas* suspensions, the five- and the three-species suspensions showed least growth. Because *Pseudomonas* is common to these three least growth-promoting suspensions, there may be some effect of this species acting to deter division. More complete

study of the effect of *Pseudomonas* in mixed-population suspensions would be needed to reveal its symbiotic role; that is to say, the possible maximum or optimal numbers of *Pseudomonas* in a *Euglena* culture are not known from this limited survey.

Deterrence of division-rates was so universal as to lead one to suspect that, in the salt solution which was used, the presence of these bacteria react on *E. gracilis* adversely rather than beneficially. It is clear from these experiments that in the absence of some organic material, which is present in the wheat infusion, *Euglena* grows better in salt solution in the absence of bacteria than in the presence of the bacteria studied. The end-products of bacterial activity may not be absorbed, and the disintegration substances of dying and/or dead bacteria may be sufficient to limit division of *Euglena*.

In any event, as the numbers of introduced bacteria become smaller and conditions therefore approach those in sterile salt solutions, the effects of differential inoculation became similar to those found with the cultures in sterile salt solutions.

Also, whatever the true relationships are between *E. gracilis* and its associated bacteria in salt and wheat cultures, it is clear that in salt solutions and in the less concentrated cultures of many different types of bacterial suspensions, greater numbers in the inocula were more protective and effected greater survival. In the more dense suspensions the smaller numbers in the inocula sometimes show significantly greater numbers surviving. This latter effect may be related to the spatial factor found elsewhere in aggregation phenomena but not indicated especially in this population study heretofore. The former effect may be due to the greater capacity of increased numbers to counteract the toxicity of such bacterial suspensions in salt solution and of some of the toxic effects of plain salt solutions in large volumes of cultures made relatively free from bacteria by dilution methods as reported in Section III and by irradiation as shown in Section IV.

VII. THE EFFECTS OF NUMBERS ON SURVIVAL OF *Euglena gracilis* SUBJECTED TO ULTRA-VIOLET IRRADIATION

The methods employed for these cultures was that of transferring the desired number of euglenae after 20 minutes in dilution Bath C into open depression slides in 4 drops of sterile wheat solution, or bacteria-rich wheat solution, or into 1 or 4 drops of sterile salt solution. They were then subjected, as was described previously, for varying lengths of time to the same type of ultra-violet irradiation. As usual, results are based on counts made at the end of 24 hours. The solution for the sterile wheat was prepared by using boiled wheat after it had stood 24 hours, as described in Section II. The bacteria-rich wheat suspensions were similar to the type used in Section V with 1 cc. on the supernatant fluid of centrifuged *Euglena* wheat infusions mixed with 10 cc. of sterile wheat solution.

The protection of numbers of *E. gracilis* is apparent in the differences with which individuals are able to withstand the deleterious effects of long exposure to ultra-violet rays (Fig. 5). The presence of organic compounds and of bacteria definitely contributes to the survival-time of *E. gracilis* in sterile and bacteria-rich wheat infusions, particularly when ten euglenae are present and when twenty and forty are present through 6 minutes' exposure; beyond that time this differential was not present.

Cultures with single individuals in 4 drops of any of the three solutions were all killed after no more than 4½ minutes' exposure. Those with ten euglenae survived the

6½ minutes' exposure in sterile wheat and bacteria-rich wheat solutions, whereas those in sterile salt solution survived no longer than did isolated individuals. Cultures with twenty individuals were able to live in the sterile salt solution after a 7-minute exposure; and in the wheat, sterile, and bacteria-rich, after an 8-minute exposure. Forty euglenae in 4 drops withstood exposures of 7½ minutes in sterile and bacteria-rich wheat solutions; in sterile salt solution the majority died at the end of 6 minutes' exposure, although a few persisted after 8 minutes. This is the only medium which allowed such survival after long exposure.

When euglenae are exposed in sterile salt-solution cultures of only 1 drop, the dele-

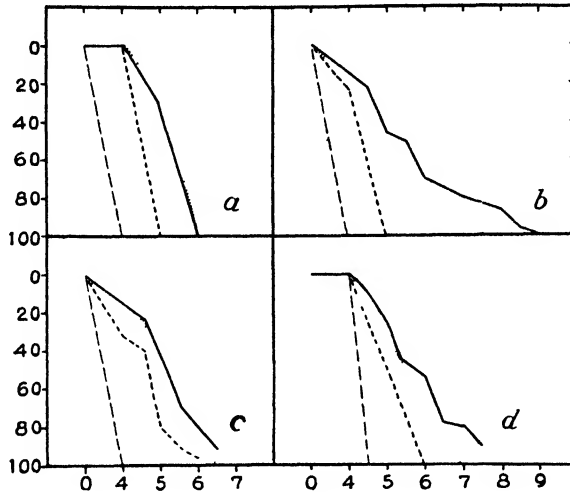


FIG. 5.—Average numbers of *Euglena gracilis* present after 24 hours, in three types of culture media, after exposure to ultra-violet irradiation of varying durations. (a) One drop, sterile salt solution. (b) Four drops, sterile salt solution. (c) Four drops, sterile wheat solution. (d) Four drops, bacteria-rich wheat suspension. Ordinates, percentage of loss; abscissas, duration of irradiation in minutes. . . . = single euglenae; - - - = ten euglenae; - - = twenty euglenae; — = forty euglenae.

terious effect of irradiation is greatest; and only cultures with twenty and forty organisms survive a 6-minute exposure; those inoculated with one or ten, only 4 minutes.

This small group of comparisons shows the advantage afforded greater numbers in any of these media in withstanding the effects of long exposure to ultra-violet irradiation and the relatively beneficial effects of organic media during all except the longer exposures. In the cases of twenty and forty euglenae in the bacteria-rich media, the presence of bacteria as an added protection against the toxicity of the rays is apparent. In the smaller volume of sterile salt solutions the deleterious effects are more drastic; even in these an increase in numbers of euglenae present insures more protection.

These relations cannot be explained on the basis of the amount of penetration, in so far as volume and bacterial effects are concerned. The presence of one or of forty euglenae in 1 or 4 drops of media, with or without bacteria or their end-products, would make very small difference in the penetration of the ultra-violet rays; hence, this effect is probably caused by some other set of relationships. Since the euglenae were cultured in the same solutions in which they were exposed to ultra-violet irradiation and since

irradiated water normally has an increase in ozone, it may well be that we are dealing here simply with mass protection from ozone. Euglenae are not particularly favorable organisms for a further analysis of the mechanism of this protection. Eliminating ozone as a factor, using the planarian, *Euplanaria dorocephala*, Allee and Wilder (1939) have demonstrated mass protection from ultra-violet irradiation and have made some progress with an analysis of protective factors.

VIII. GENERAL DISCUSSION AND SUMMARY

Cultures in inorganic salt solution, almost or quite bacteria-free, in various suspensions of bacteria, in sterile and bacteria-rich wheat infusions, and of volumes ranging from 1 to 40 drops, have been used in this study of populations of *E. gracilis*. With numbers present at the end of 24 hours as an index, a variety of differences in division-rates of cultures inoculated with one, two, four, and eight individuals have been analyzed in order to find the effect of numbers used in initial seedings on survival and population growth. Constancy of light, temperature, humidity, pH, and in most cultures a strictly autotrophic media have been maintained. The controlled variables include, in addition to the number of euglenae inoculated, kinds and numbers of bacteria, volume, including surface area, and two methods of securing bacteria-free cultures. All the individuals used have been members of two clones between which and within which no measured differences in physiological states or division rates have been significant.

With such relatively small subcultures the results obtained indicate:

1. Greater rate of growth with smaller inocula:
 - a) Washed-sterile euglenae in 1 (0.05 cc.) to 4 (0.20 cc.) drops
 - b) Irradiated-sterile euglenae in 4 drops
2. Greater maintenance with smaller inocula:
 - a) In bacterial suspensions
 - (1) 0.01 X concentration, *Staphylococcus* suspensions
3. Greater survival with smaller inocula:
 - a) In more concentrated bacterial suspensions
 - (1) X concentration, five-species suspensions
 - (2) 0.1 X concentration five-species suspensions
 - (3) 0.1 X concentration
1. Greater rate of growth with larger inocula:
 - a) Washed-sterile euglenae in 5 (0.25 cc.) drops
 - b) Irradiated-sterile euglenae in 1 drop
 - c) Nonsterile cultures of 1 drop
2. Greater maintenance with larger inocula:
 - a) In bacterial suspensions
 - (1) 0.01 X concentration, *Flavobacterium* suspensions
3. Greater survival with larger inocula:
 - a) Washed-sterile euglenae in 10, 20, and 40 drops
 - b) In more concentrated bacterial suspensions
 - (1) 0.01 X concentration three-species suspensions
 - (2) 0.01 X concentration, *Flavobacterium* suspensions
 - (3) 0.01 X concentration, *Pseudomonas* suspensions
 - c) In less concentrated bacterial suspensions
 - (1) 0.001 X concentration, *Pseudomonas* suspension
 - (2) 0.0001 X concentration *Staphylococcus* suspensions

- (3) 0.0001 X concentration *Flavobacterium* suspensions
- (4) 0.0001 X concentration, three-species suspensions
- d) With exposure to toxic durations of ultra-violet irradiation
 - (1) In 1 and 4 drops of sterile salt solution
 - (2) In sterile wheat solution
 - (3) In bacteria-rich wheat suspensions

From these results in bacteria-free cultures it is apparent that density of inocula markedly affects growth and/or survival of populations of *E. gracilis*. Also, it seems clear that there exist optimal, suboptimal, and supraoptimal volume relations in cultures with washed-sterile euglenae which regulate growth or survival, and, also, that there are optimal, suboptimal, and supraoptimal densities of the bacteria associated with *Euglena*. For the most part, the mere presence of bacteria in a salt solution deters the development of euglenae. However, some materials or material are present in wheat media, even when autoclaved, which is accelerative to division-rates and which also affords protection against the toxic effects of ultra-violet irradiation.

Frequently during the experiments reported here, the numbers of individuals in the inoculum of a culture have made a significant difference in the rates of division and abilities to withstand environmental exigencies. As the foregoing summary shows, the smaller inocula have the advantage in a number of instances: with washed-sterile euglenae in 1 to 4 drops regardless of identical volume relations; in the cultures with larger volumes started with irradiated euglenae; and in the more concentrated bacterial suspensions. Stimulation due to the absence of members of the same species, and/or absence of a larger quantity of overly toxic substance or substances or of deleterious physical conditions, may determine the reactions of smaller numbers in the first instances. With the denser bacterial suspensions it is possible that survival is greater because of less competition in these more-dense mixed populations for space, light, acids, gases, or for certain of these.

Instances in which a larger inoculum has significant advantages over smaller inocula appear to be more numerous under the conditions tested. When the defects of an inorganic media accumulate, as in the volumes of from 5 to 40 drops; when toxic effects of irradiation are increased in smaller volumes, owing to greater evaporation, etc., as in the 1-drop versus 4-drop cultures; when time of exposure to ultra-violet irradiation is increased to a toxic amount; and when moderate and more dilute concentrations of various species of bacteria are present, greater numbers in the inocula insure cultures of having greater reproductive rates or greater chances of survival, or both.

There is no evidence from these experiments to indicate whether the irradiated-sterile, the washed-sterile, or nonsterile euglenae are most nearly normal. We know that in nature *Euglena* is an autotroph of stagnant, fresh-water, unshaded pools. In view of the type of reaction which occurs in the presence of mixed bacteria (of these relatively dense concentrations) and in the presence of a toxic agent (as represented by irradiation), protection as a result of the presence of greater numbers in the inoculum undoubtedly could occur in nature. Likewise, in the absence of bacteria, and with too great toxicity of the media, the accelerated division-rate of reduced numbers in the in-

oculum of *E. gracilis* may occur. That the types of population physiology shown in these cultures may exist in nature in a similar manner and not be limited to a few laboratory setups is not, therefore, unreasonable.

The experiments were not planned, however, to duplicate nature. They were conducted in the manner here recorded in an effort to bring the complex conditions to be found in an ordinary laboratory infusion under at least partial experimental control. These analytical experiments were attempted only after a long series of tests with more complex, and therefore less closely controlled, conditions which yielded indefinite, erratic results.

There have been described in this paper a number of situations, but there has been no attempt to analyze the causal factors at the base of such relationships as exist in these relatively simple experimental populations. The results of this study are relatively consistent within themselves and with the findings of others. Some of the complexities involved in this paper are, a priori, confusing. Errors in reasoning and in the conclusion based on this work may possibly be due to the natural tendency to simplify the categories and so avoid apparent inconsistency, in place of allowing many observations to dangle unrelated.

Similar work with protozoans has been done mainly with ciliates; and the relations obtaining in members of that group, fully discussed and reviewed by Allee (1931, 1934, 1935) and Johnson (1937), do not necessarily hold with an autotrophic organism. A discussion of the similarities and differences in results dependent on different physiological systems is reserved for the present.

The recent studies of Mast and Pace (1938) with the flagellate, *C. paramecium*, and of Reich (1938) with a soil amoeba, *M. palestinesis*, present additional substantiation of an allelocatalytic effect, whatever its basic factors may be. According to Reich (1938, p. 357), "cultures whose initial population was less than 0.03 amoebae per 0.1 cu. mm. of solution showed a distinctly lower rate of division in the first days than the controls with higher initial populations." And Mast and Pace (1938, p. 380) find "the rate of reproduction of *Chilomonas* increases to a maximum as the volume of culture fluid per individual decreases and then decreases to zero." The results obtained in various modifications of their experiments strongly indicate that *Chilomonas* secretes a substance which favors growth which they call *X*.

The only population study with a green flagellate that was available when the present paper was written was that of Jahn (1929), who found that the density of *E. gracilis* at the beginning of mass cultures which favor greatest growth was to be found in initial concentrations ranging from 38 to 39 organisms per cubic centimeter, rather than in those with a greater density of 350 to 894 organisms per cubic centimeter. In his work it is clear that "fewer" numbers in the inocula are most favorable in the early development of large quantity *Euglena* cultures.

It may be said that the conditions of his experiments were so different from those herein described as to make a comparison of results impossible.⁶ And so it may be, but it is interesting to note certain similarities in results which do appear. If we analyze the terms "small" and "large" seedings in both series, we find a basis for comparison which may shed some light on Jahn's results and those of the present work.

⁶ The composition of the media used by Jahn contained hydrolyzed casein and different inorganic salts; surface-area relations of mass cultures are different from those of depression-slide cultures.

In the present study of small-volume cultures the greatest concentration of organisms inoculating a culture was that of eight individuals in 1 drop, which is approximately 160 individuals per cubic centimeter. The concentrations which yielded greatest rates were those of one and two individuals per 4 drops (or five and ten organisms per cubic centimeter) and those of four and eight individuals per 5 drops (or sixteen and thirty-two organisms per cubic centimeter). The most dilute concentration of initial population was one organism per 40 drops or one-half organism per cubic centimeter. In other words, the greatest division-rates obtained in this range of small-volume cultures occurred in the moderately dense seedings. A comparison of Jahn's findings with a part of the data at hand (those with washed-sterile euglenae in salt solutions) is possible in this form.

- | | |
|--------------------------------------|--|
| A. Jahn's mass cultures | <p>Cases in which less concentrated inocula gave a greater division-rate than more concentrated:</p> <ol style="list-style-type: none"> 1. 89 individuals per cubic centimeter, compared with 890 individuals per cubic centimeter, showed a 10:1 division-rate 2. 55 individuals per cubic centimeter, compared with 555 individuals per cubic centimeter, showed a 3:1 division-rate |
| B. Present depression-slide cultures | <p>Cases in which less concentrated inocula gave a greater division-rate than did more concentrated inocula:</p> <ol style="list-style-type: none"> 1. 20 individuals per cubic centimeter (one individual per 1 drop), compared with 160 individuals per cubic centimeter (eight individuals per 1 drop), showed a 12.6:9.8 division-rate 2. 5 individuals per cubic centimeter (one individual per 4 drops), compared with 40 individuals per cubic centimeter (eight individuals per 4 drops), showed a 27.0:16.0 division-rate <p>Cases in which more concentrated inocula gave a greater division-rate than did less concentrated inocula:</p> <ol style="list-style-type: none"> 1. 160 individuals per cubic centimeter (eight individuals per 1 drop), compared with 8 individuals per cubic centimeter (eight individuals per 20 drops), showed a 9.8:3.25 division-rate 2. 40 individuals per cubic centimeter (eight individuals per 4 drops), compared with 4 individuals per cubic centimeter (four individuals per 20 drops), showed a 10.3:1.25 division-rate |

By combining the present findings with those of Jahn, it is possible to suggest that a supraoptimal density of initial populations of washed-sterile *E. gracilis* ranges above 80 individuals per cubic centimeter. An optimal size of inoculum apparently lies between 4 and 50 individuals per cubic centimeter, and a suboptimal one contains still fewer euglenae. This is a broad optimal range, to be sure; and it would not hold in each specific detail, particularly in depression-slide cultures, because there is a definite function of particular volumes, with an optimum at 4 and 5 drops in these experiments, regardless of initial population density. Jahn did not vary the surface area or volume of his mass cultures, so that relations which may depend on these are not brought to light.

Such a comparison as this indicates, at least, the necessity of using the terms "larger" and "smaller" with caution when referring to size of inocula, and of using, rather, the number of individuals per cubic centimeter wherever possible in drawing generaliza-

tions. In short, the densities of inocula which Jahn terms "light" are in the present work "heavy"; and the greatest division-rates occurred with approximately the same densities of initial population in both Jahn's mass cultures and the present series, in which small volumes are used.

Other functions or reactions of different numbers of the same species in the seeding of a culture appear in several ways in the data from small subcultures.

The data, as a whole, add a greater number of instances to the rapidly growing amount of verification of the principle of automatic co-operation among all plant and animal life than to the older and generally more easily proved interorganismic effects associated with overcrowding. Thus, these experiments lend additional support to the idea of the existence of an automatic mutualism, which Allee (1938) and his associates are investigating.

That signs of the beginnings of automatic co-operation and, to that extent, of a meager social organization appear in these unicellular and solitary chlorophyll-bearing organisms, whose status as members of the plant or animal kingdom is still in dispute, is not strange in view of the fact that colonial organization and even sexual differentiation are also to be found in the closely related Phytomonadida.

IX. CONCLUSIONS

1. Optimal, supraoptimal, and suboptimal volume relations of cultures of washed-sterile euglenae exist, which regulate growth or survival of a group accordingly.

2. There are optimal, suboptimal, and supraoptimal densities of bacteria associated with euglenae populations. Deterrence of division is common in the densities and with the species of bacteria tested.

3. Some material or materials present in sterile or nonsterile wheat infusions is more conducive to growth of populations of euglenae, and also affords more protection against toxic effects of ultra-violet irradiation, than does the artificial salt solution tested.

4. Smaller inocula show significantly greater advantage in situations in which the deleterious elements of the environment are not too great.

5. Larger inocula show significantly greater advantage when the defects of the environment are greatest.

6. Jahn (1929) found, from the study of mass cultures, no evidence of the Robertson effect; his least-dense cultures, however, correspond fairly well to the optimal numbers constituting an inoculum for the present small volume cultures, whose division-rates were found to be greater than either those in more dense or more dilute cultures.

7. The Robertson effect, or certain aspects of it, is present in some of the optimal volumes and may be operating in all of the supraoptimal volumes reported in this paper.

8. To this extent the present studies yield evidence of Robertson's effect. There is, however, no clear evidence of the validity of his theory.

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FLUCTUATIONS IN GROWTH-RATE OF *EUGLENA ANABAENA*, *E. GRACILIS*, AND *E. VIRIDIS*, AND THEIR APPARENT RELATION TO INITIAL DENSITY OF POPULATION

(Six figures)

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IN STUDIES on autotrophic strains of *Euglena anabaena*, *E. gracilis*, and *E. viridis* in inorganic media the writers noted marked changes in the rate of population growth in successive transfers. In view of Dusi's (1937) observations that constant light was unfavorable for growth of certain Euglenidae, all our experimental lines were maintained at room temperature near an east window, and each transfer was incubated for 3 weeks. Although external conditions were fairly uniform for the different experimental series at any given time, both light and temperature varied from day to day. Since temperature and light were thus not constant during successive periods of incubation, variations in these conditions were at first suspected of inducing the fluctuations in growth-rate. This hypothesis was necessarily discarded when it was observed that the growth-rate was increasing in certain lines while decreasing in others. The pH of the medium was also eliminated as an important factor, since marked differences in growth-rates were observed in successive transfers showing the same initial and final pH values. The initial density of population also varied in successive transfers and it was noted that this factor showed certain relations to the growth-rate. A similar relationship has been described previously by Jahn (1929), who demonstrated that, in a pure line of *Euglena* sp., the rate of population growth was inversely proportional to the initial concentration of flagellates, particularly in cultures incubated for 10 days or longer. The observations described below are in general agreement with the findings of Jahn and show further that this relationship may be important in interpreting growth of a line through a series of successive transfers. The relationship between initial density of population and growth-rate has been traced in five different autotrophic lines, two of *E. gracilis*, one of *E. anabaena*, and two of *E. viridis*.

MATERIAL AND METHODS

Euglena anabaena var. *minor* (referred to here as "*E. anabaena*") and *E. gracilis*, in pure lines established by Mainx, were obtained bacteria-free from Professor E. G. Pringsheim in 1931; and a Mainx strain of *E. viridis* was obtained from the same source in 1937. Autotrophic strains of the three species have been established (Hall, 1938, 1939; Hall and Schoenborn, 1939) in inorganic media and have been utilized in the observations described below.

The following inorganic media were used:

MEDIUM EAB		MEDIUM EF	
NH ₄ NO ₃	0.5 gm.	NH ₄ NO ₃	1.0 gm.
KH ₂ PO ₄	0.5 gm.	MgSO ₄ ·7H ₂ O.....	0.2 gm.
MgSO ₄ ·7H ₂ O.....	0.1 gm.	CaCl ₂ ·2H ₂ O.....	0.1 gm.
NaCl.....	0.1 gm.	KH ₂ PO ₄	0.2 gm.
FeCl ₃ ·6H ₂ O.....	0.0025 gm.	FeCl ₃ ·6H ₂ O.....	0.0025 gm.
MnCl ₂ ·4H ₂ O.....	0.0001 gm.	MnCl ₂ ·4H ₂ O.....	0.0001 gm.
Distilled water.....	1.0 liter	Distilled water.....	1.0 liter

In allowing for the drop in pH during sterilization the reaction of each medium was adjusted with NaOH to pH 7.3–7.7. The medium was then tubed in measured amounts and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

In the first transfer of every experimental series each tube received a 0.5-cc. inoculum from an autotrophic culture of the appropriate species. The tubes of the second transfer were inoculated in the same way from a tube of the first transfer after incubation, and so on in subsequent transfers. In each transfer approximately fifteen tubes were inoculated. Four of these were fixed for the initial count (number of flagellates per cubic centimeter), one was used for determination of initial pH, and the remainder were incubated for 3 weeks at room temperature near an east window. After incubation four tubes were fixed for the final count, one was used for determination of final pH, and another for inoculation of the next transfer. All pH determinations were made with a La Motte roulette colorimeter. Initial and final counts were made with a Whipple ocular micrometer and a Sedgwick-Rafter counting-cell. The amount of growth, expressed as x/x_0 (ratio of final to initial concentration of flagellates per cubic centimeter), was determined by a comparison of the final and initial counts.

GROWTH OF *Euglena gracilis*

Series I in medium EF was followed for 33 weeks. Tubes of the first transfer were inoculated from an autotrophic culture of *E. gracilis* in the same medium at pH 6.6. The initial pH in the eleven successive transfers was: 6.9, 6.9, 6.9, 7.3, 6.9, 6.7, 6.9, 7.5, 6.9, 6.9, and 6.6. Each transfer was incubated for 21 days. Initial counts, growth, and final densities of population in the different transfers are compared in Figure 1. It will be noted that, for any two successive transfers except the sequence 7–8, a rise or fall in initial count is reflected inversely in the x/x_0 values. Transfer 8 showed both a low x/x_0 value and a small final population. These results may have been caused by a high initial pH in combination with a period of cold, cloudy weather in which the laboratory temperature was lower than that of previous periods.

Series II in medium EAB also covers a period of 33 weeks. The first transfer was inoculated from an autotrophic strain of *E. gracilis* in medium EAB at pH 6.7. Initial pH in successive transfers was as follows: 6.8, 6.9, 6.8, 6.7, 6.8, 6.7, 6.9, 7.0, 6.9, 6.9, and 7.1. The initial counts, x/x_0 values, and final densities of population are compared in Figure 2. The inverse relationship between growth and initial density of population is obvious in successive transfers.

Series III in medium EF was designed to compare cultures with different initial counts in regard to the relation between initial density of population and the amount of growth. In order to obtain a high initial concentration the contents of two tubes from transfer 8

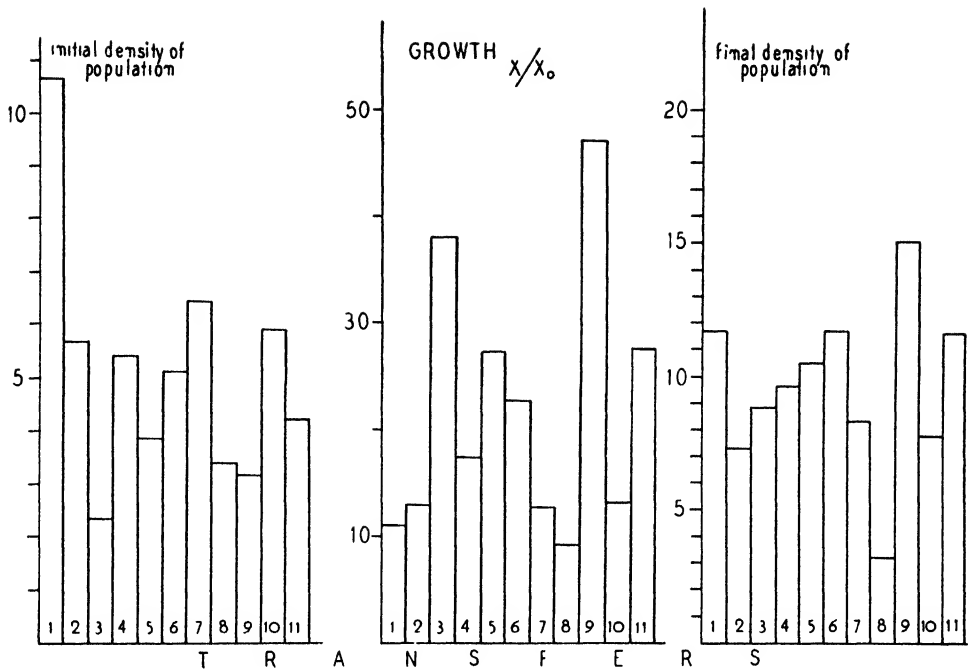


FIG. 1.—*Euglena gracilis*, series I. Initial density of population (hundreds per cubic centimeter), amount of growth (x/x_0), and final density of population (thousands per cubic centimeter) in eleven successive transfers.

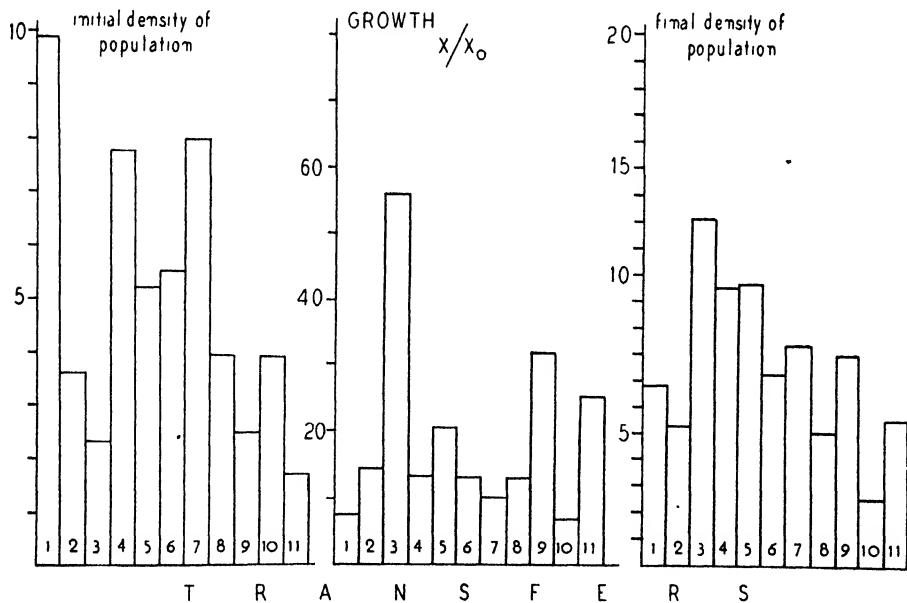


FIG. 2.—*Euglena gracilis*, series II. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) in eleven successive transfers.

of series I were combined and used for inoculation (1.0 cc. of inocula) of the first transfer in series III. This transfer was inoculated at the same time as transfer 9 of series I and was identical with the latter with respect to type and pH of medium and time and other conditions of incubation. Any difference in rate of growth could, therefore, be attributed to the difference in inocula. In the second transfer, inoculations of 0.8 cc. were made from a tube of transfer 9 (ser. I); in all other respects transfer 2 (ser. III) was comparable to transfer 10 (ser. I). In the third transfer 2.0 cc. of material from a series I (transfer 11) tube were added to a tube of medium EF, which was used for inoculation (0.5 cc. of

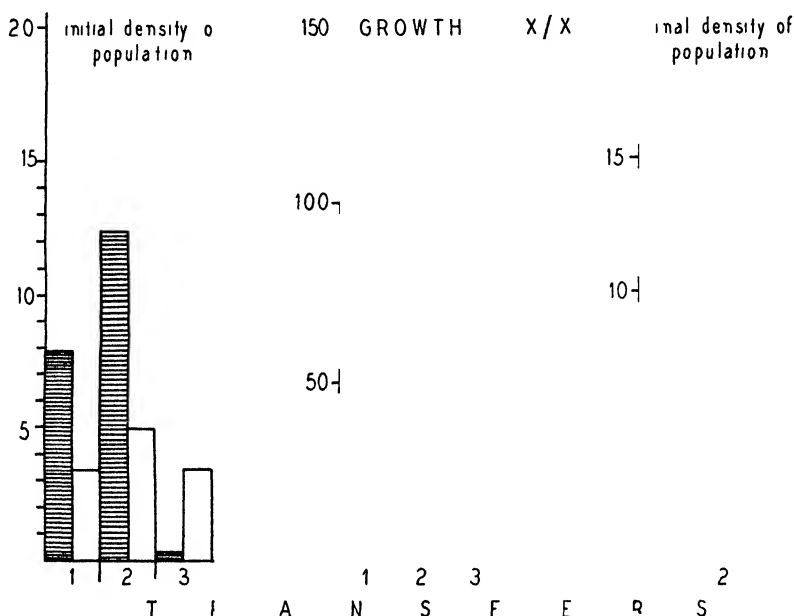


FIG. 3.—*Euglena gracilis*, series III. Transfers in series III (cross-hatched blocks) are compared with corresponding transfers in series I (white blocks) with respect to initial density of population (hundreds per cubic centimeter), growth (X/X_0), and final density of population (thousands per cubic centimeter).

inocula) of transfer 3 in series III. This procedure reduced the initial count for the third transfer to approximately one-fourteenth that for the corresponding transfer of series I. In Figure 3 the three transfers of series III are compared with the corresponding transfers of series I with respect to initial density of population, growth (X/X_0), and final density of population. In each transfer the inverse relationship between initial count and growth is apparent.

GROWTH OF *Euglena anabaena*

Series IV in medium EF covers a period of 24 weeks. The first transfer was inoculated from an autotrophic strain of *E. anabaena* in medium EF at pH 6.9. The initial pH in successive transfers was as follows: 6.9, 7.3, 6.9, 6.7, 6.9, 7.5, 6.9, and 6.9. In this series (Fig. 4) an inverse relationship between initial count and growth is apparent in every transfer.

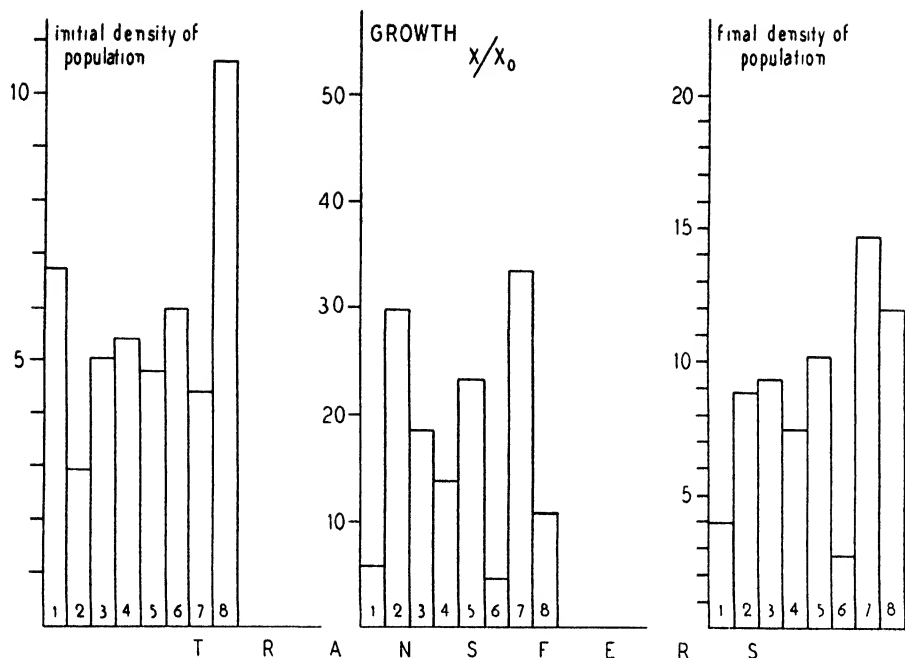


FIG. 4.—*Euglena anabaena*, series IV. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) are compared in eight successive transfers.

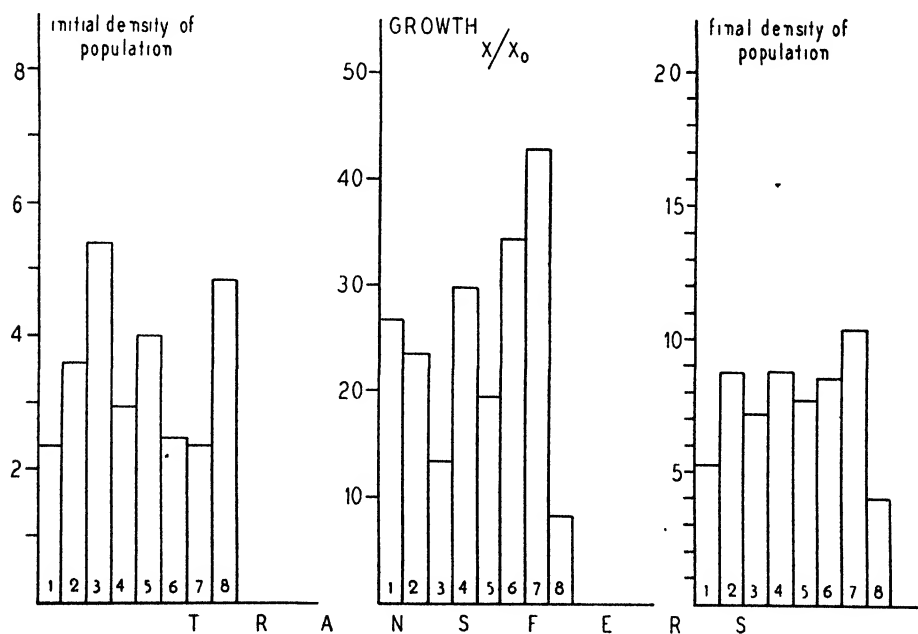


FIG. 5.—*Euglena viridis*, series V. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) in eight successive transfers.

GROWTH OF *Euglena viridis*

Series V in medium EF was run concurrently with series IV. The first transfer was inoculated from an autotrophic strain of *E. viridis* in medium EF at pH 6.9. Initial pH in successive transfers was: 6.9, 7.3, 6.9, 6.7, 6.9, 7.3, 6.9, and 6.9. This series (Fig. 5) also showed inverse relationships between initial density of population and growth (x/x_0).

Series VI in medium EAB was carried for 24 weeks. Tubes of the first transfer were inoculated from a strain of *E. viridis* in medium EF at pH 6.9. Initial pH in successive

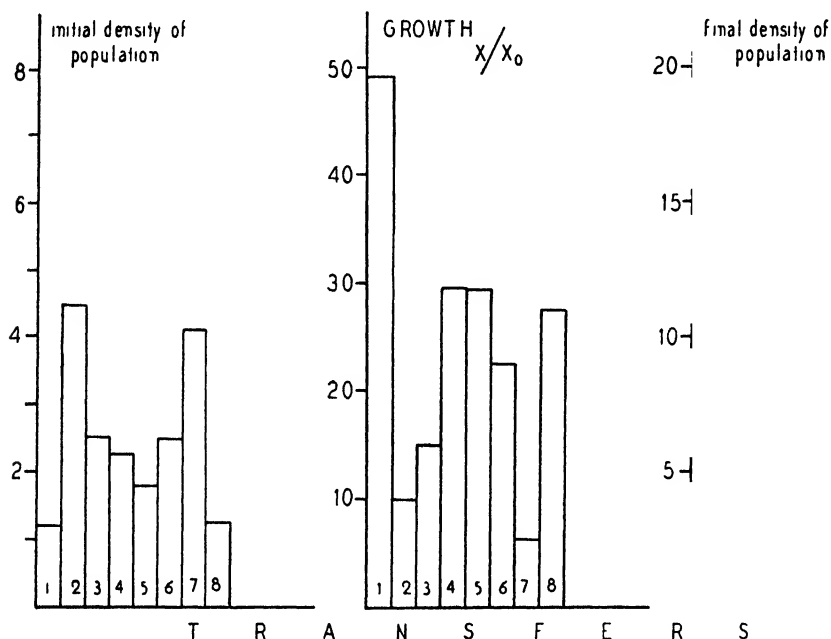


FIG. 6.—*Euglena viridis*, series VI. Initial density of population (hundreds per cubic centimeter), growth (x/x_0), and final density of population (thousands per cubic centimeter) in eight successive transfers.

transfers was: 6.7, 6.7, 6.7, 6.9, 7.0, 6.9, 6.9, and 6.9. In this series, as in the preceding ones, an inverse relationship between growth and initial count in successive transfers is apparent (Fig. 6) except in transfer 5. The x/x_0 values were practically identical in transfers 4 and 5, whereas greater growth was to have been expected in transfer 5. This fifth transfer overlapped in time transfer 8 in series I, where similar anomalies were observed.

DISCUSSION

On the basis of available information concerning growth of protozoan populations, it may be assumed that a population of *Euglena* in an inorganic medium increases to a certain maximal density (maximal stationary phase of growth) and then begins to decline. Preliminary observations have indicated that a population of *Euglena*, in our media, may approach the maximal stationary phase in cultures incubated for 3 weeks under favorable conditions. Under suitable environmental conditions a given culture

may approach the maximum quite closely, while a culture exposed to less favorable conditions will fall short of the maximum in a comparable growth period. Thus, differences in final population density were to be expected in different transfers of our experimental series, since the transfers were subjected to different environmental conditions. Such differences were observed (Figs. 1, 2, 4-6) and are probably to be attributed, in large measure, to variations in temperature and in intensity of light, factors obviously influencing the rate of population growth.

While environmental factors are undoubtedly important in determining the final density of population, it does not follow necessarily that the growth-rate is dependent upon these factors alone. Since the growth-rate (x/x_0), as the term is used here, represents the ratio of final to initial density of population, the initial density must also be considered as a factor. In our technique, as described above, the initial density of population for any given transfer is approximately one-twentieth the final density of population in the preceding transfer. Consequently, a large final population in one transfer would be followed by a high initial count in the next transfer, and a smaller population by a lower initial count.

The final density of population is obviously a function of the initial density and the growth-rate. If the rate of growth is approximately the same for a given set of environmental conditions, it must follow that a high initial count will give rise to a large final population and a lower initial count to a smaller population. However, there is no such correlation between initial and final densities of population in the results (Figs. 1-6) described above. As an alternative hypothesis, it may be supposed that, under the conditions of our experiments, a population of *Euglena* will approach the maximal stationary phase of growth, whether the initial count is comparatively high or comparatively low. If this is true, the growth-rate (x/x_0) will show an inverse relationship to the initial density of population.

The second hypothesis was tested in series III, in which paired transfers, differing only in inocula, were compared with respect to growth-rate and final density of population. In the first transfer (Fig. 3) the ratio of initial densities of population was 2.43:1; the corresponding growth-rates showed the inverse ratio of 1:2.5; and the final densities of population differed by 6 per cent, an amount not statistically significant. In the second transfer the corresponding ratios were 2.23:1 and 1:2.31, while the final populations differed by only 3.7 per cent. In the third transfer the ratios were 1:14.2 and 10.9:1. In this case, with a difference in initial counts much greater than in the other two transfers, the inverse relationship between initial density of population and growth-rate is less exact, although still apparent. The final populations (Fig. 3) in the various transfers differed appreciably; but in each transfer comparable populations were obtained in the paired series, irrespective of the initial count. These results indicate that for a given set of conditions, as specified above, the final density of population is, to a large extent, independent of the initial density, while the rate of growth shows an inverse relationship to the latter.

With due allowance for the fact that successive transfers in the various series were incubated at different times and hence were subjected to different conditions of light and temperature, the inverse relationship between growth and initial density of population should hold in all our experimental series. Such inverse relationships are apparent. For example, in successive transfers of series II these ratios are as follows: first and second transfers, 2.7:1 and 1:2.1; second and third, 1.5:1 and 1:3.8; third and fourth, 1:3.3 and

4.1:1; fourth and fifth, 1.5:1 and 1:1.5; fifth and sixth, 1:1.2 and 1.5:1; sixth and seventh, 1:1.5 and 1.3:1; seventh and eighth, 2.0:1 and 1:1.3; eighth and ninth, 1.6:1 and 1:2.4; ninth and tenth, 1:1.5 and 4.9:1; and tenth and eleventh, 2.2:1 and 1:4.0. With two exceptions, noted above (ser. I, transfer 8; and ser. VI, transfer 5), comparable relationships are evident in the other series. In general, when the final densities of population in successive transfers were approximately the same, the inverse relationship between growth-rate and initial density of population approached mathematical exactness. Several of the best examples are as follows: series I, fourth and fifth transfers, 1.4:1 and 1:1.5, and fifth and sixth transfers, 1:1.3 and 1.2:1; series II, fourth and fifth, 1.5:1 and 1:1.5; series IV, second and third, 1:1.7 and 1.6:1; series V, fifth and sixth, 1.6:1 and 1:1.7; and series VI, fifth and sixth, 1:1.37 and 1.28:1.

It is, of course, obvious that the growth-rate of *Euglena* in inorganic media is controlled in part by temperature and intensity of light, and it is important to determine whether variations in these factors could be primarily responsible for the fluctuations in growth-rate. This question may be tested by comparing the growth-rates in transfers of different series incubated during the same periods and under the same conditions. Thus, transfers 3-10 of series I correspond to transfers 1-8 of series IV and V. Similarly, transfers 4-11 of series II correspond to transfers 1-8 of series VI. A comparison of the corresponding growth-rates (Figs. 1, 2, 4-6) shows no correlation which can be attributed primarily to environmental factors. Hence, it appears that, even in transfers incubated at different times and under somewhat different environmental conditions, the initial density of population is more important in its influence on growth-rate than are various environmental factors which in themselves are known to affect the rate of population growth.

Such a relationship between growth-rate and initial density of population was clearly shown by Jahn (1929) in certain experiments with *Euglena* sp. in an inorganic medium, although his cultures were not bacteria-free. Our observations with bacteria-free cultures are in agreement with these findings of Jahn and show further that variations in initial density of population may bring about fluctuations in growth-rate of a strain during successive transfers in a given medium. Such fluctuations are, to a large extent, independent of environmental factors.

SUMMARY

Fluctuations in growth-rate of autotrophic strains of *E. anabaena* var. *minor*, *E. gracilis*, and *E. viridis* were noted in successive transfers in inorganic media. These fluctuations appear to be dependent upon an inverse relationship between growth-rate and the initial density of population. Under the conditions described, the initial density of population seems to be more important, in its influence on growth-rate, than are environmental factors which in themselves influence the rate of population growth.

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THE EFFECT OF TEMPERATURE ON THE SIZE OF THE COLUMNAR CELLS OF THE MID-INTESTINE OF THE JAPANESE BEETLE LARVA (POPILLIA JAPONICA NEWMAN)

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ACCORDING to Titschack (1925), it is a general rule that insects reared at low temperatures grow to a larger size than those reared at higher temperatures. He discusses a few cases in which the reverse condition exists, and attributes the smaller size attained at lower temperatures to factors other than temperature. In many cases geographic races of a species exist in which the northern forms have been found to be smaller than southern forms of the same species. Titschack explains these cases as due either to differences in food eaten by the larvae or to a recent introduction of the species to a northern range in which temperature conditions are no longer optimal for development. Bělehrádek (1935), in his monograph on *Temperature and Living Matter*, discusses the effects of temperature on the size of cells and their constituents. He reviews about twenty papers recording observations on Protozoa, higher animals, and plants; and in every case larger cells were reported as found in organisms grown at lower temperatures.

Ludwig (1932) studied the growth of Japanese beetle larvae at temperatures of 20° and 25° C. He found that at all corresponding stages of development individuals were smaller when grown at the lower temperature. This smaller size of larvae, pupae, and adults at 20° might be associated with a smaller number of cells; or the cells themselves might be smaller in individuals grown at the lower temperature. In the present study, to determine which of these factors is responsible for the observed difference in size, the cells in the region of the mid-intestine limited by the second and third rings of gastric caeca were counted and measured in larvae grown at 20° and 25° C. These cells were selected for study because Abercrombie (1936) reported that, during the growth of the Japanese beetle, their increase in volume is proportional to the increase in size of the entire animal.

MATERIAL AND METHODS

Larvae of the Japanese beetle were obtained from eggs collected in the laboratory. As soon as hatched, they were isolated in 1-ounce metal salve boxes containing, as a medium, moist *Andropogon* mold. The salve boxes and the medium had been previously sterilized by heating for several hours at 150°–200° C. Several grains of wheat were placed in each box to serve as additional food for the larvae. They were then divided into two groups, one being placed in an incubator at 20° and the other at 25° C. Humidity was kept near the point of saturation by the addition of tap water to the medium; and food, in the form of both *Andropogon* mold and grains of wheat, was available for the larvae at all times.

Larvae from each group were killed at stated stages of development at which studies on cell number and cell size were desired. The stages selected were the newly hatched

larva, freshly molted second-instar larva, and freshly molted third-instar larva. These stages were selected because each is probably of an equivalent age at the two temperatures and also because, immediately following the molt, less material is present within the alimentary canal to interfere with sectioning. When each larva had reached the desired stage, it was weighed, its body wall cut, and it was then immersed in hot Bouin's fixative. It was then dehydrated and cleared by the *n*-butyl alcohol method (Stiles, 1934) and sectioned in paraffin. Serial longitudinal sections of entire larvae were made at $5\ \mu$ thickness in the case of first- and second-instar larvae and at $7\ \mu$ thickness in the case of the third-instar larvae. They were stained with Delafield's hematoxylin and eosin. The progressive method of staining was employed.

The columnar cells of the mid-intestine were counted in median longitudinal sections of both the dorsal and ventral walls from the point where the epithelium begins to turn out into the second ring of gastric caeca to the corresponding point near the third ring of gastric caeca. The cells were measured with an ocular micrometer, using a $10\times$ ocular and a 1.8-mm. objective. In each larva typical cells were measured in the anterior, middle, and posterior regions of both the ventral and dorsal walls of the region studied. All counts and measurements were made with an oil immersion objective.

OBSERVATIONS

The results of the cell counts and measurements are given in Table 1. The average volume of each cell was obtained by multiplying the height by the width by the breadth of the cell, the latter having been earlier found to be equal to its width.

TABLE 1
EFFECT OF TEMPERATURE ON THE SIZE OF THE COLUMNAR CELLS
OF THE MID-INTESTINE OF THE JAPANESE BEETLE LARVA

Stage	No. of Larvae Studied	Average Weight (Mg.)	No. of Cells in Ventral Wall	No. of Cells in Dorsal Wall	No. of Cells Measured	Average Width of Each (μ)	Average Height of Each (μ)	Average Volume of Each (Cu. μ)
20° C.								
First instar (freshly hatched).....	9	146	208	53	3.66	33.97	455.05
Second instar (freshly molted).....	12	13.1	217	344	72	6.20	30.48	1,171.65
Third instar (freshly molted).....	9	49.0	432	660	54	6.08	50.40	1,863.10
25° C.								
First instar (freshly hatched).....	10	147	208	59	3.87	29.43	440.77
Second instar (freshly molted).....	11	17.5	226	353	66	7.03	32.05	1,583.94
Third instar (freshly molted).....	9	79.7	443	693	54	7.26	45.61	2,403.99

Larvae reared at the lower temperature were smaller, the freshly molted second instars averaging 13.1 mg. at 20°, as compared with 17.5 mg. at 25° C. The corresponding weights for freshly molted third instars were 49.0 mg. and 79.7 mg. In spite of these differences in weight at the two temperatures, no significant differences were observed in the number of columnar cells in larvae of a given stage of development. An exact count of the cells in larvae reared at 20° was rather difficult because the cells were narrower and more crowded. The small differences shown in Table 1 are therefore not considered significant, since in no case do the numbers for a given stage differ by more than 5 per cent. Furthermore, the average numbers shown in this table for both 20° and 25° agree closely with those obtained at 25° C. by Abercrombie (1936) for larvae of corresponding stages of development.

Except in newly hatched larvae the columnar cells of the mid-intestine are definitely larger in individuals reared at the higher temperature. In freshly molted second-instar larvae the average cell volume was 1,171.65 cu. μ at 20° and 1,583.94 cu. μ at 25° C. These values are statistically different, since the difference is more than ten times its probable error. In freshly molted third-instar larvae the average volume of each cell was 1,863.10 cu. μ at 20° and 2,403.99 cu. μ at 25° C. In this case the difference is also more than ten times its probable error. In newly hatched larvae the columnar cells are approximately the same in size at both temperatures. This result was anticipated, since Ludwig (1932) found that the newly hatched larvae were the same in size, averaging 2.3 mg. at both temperatures.

The differences in size of the columnar cells of larvae reared at 20° and 25° C. are roughly proportional to the differences in weight of the entire animal. The newly molted second-instar larva at 25° weighed 1.3 times as much as at 20°, and the ratio of the cell volumes was also 1.3. For the newly molted third-instar larvae the ratio between the weights attained at the two temperatures was 1.6, and for cell size it was 1.3. Hence, the increase in size of larvae reared at the higher temperature is due, to a large extent, to the larger size of their constituent cells.

DISCUSSION

These observations and those of Ludwig (1932) indicate that the Japanese beetle is an exception to the general rule, enunciated by Titschack (1925), that insects attain a larger size when reared at a lower temperature. His explanations of exceptions to the rule do not seem to apply to this species. Titschack believed that, if a species grew to a smaller size at lower temperatures, some factors other than temperature are concerned. Among these, nutrition was considered very important. However, in these experiments with Japanese beetle larvae, food was carefully controlled, an abundance of wheat grains and *Andropogon* mold being available at all times at both temperatures. Titschack suggested that insects reared in the cold eat more food; and of the food eaten, more is absorbed and used for tissue synthesis. He performed some very exact experiments with the clothes moth, *Tineola biselliella*, which showed that at lower temperatures this species actually consumed more food and less fecal material was eliminated. While no exact comparison was made of the amount of food consumed by Japanese beetle larvae at 20° and 25° C., it was found that food had to be added much more frequently at the higher temperature. Because of the existence of a larval diapause, there is a relatively small difference between the duration of the larval stage at 20° and at 25° C. Ludwig (1928), under conditions similar to those in the present experiment, found its duration

at these temperatures to be 170 and 139 days, respectively. Hence, it is highly improbable that the amount of food consumed at the lower temperature would be greater.

Titschack believed that the recent spread of a species to a more northern climate might account for the smaller size of its northern races, since they are subjected to temperatures which are not favorable for development. If this idea is correct, we could divide insects into two groups, depending on their temperature reactions. One group includes northern species, which are adapted to cold climates and grow to a larger size when exposed to low temperatures; the other includes tropical or subtropical species, which are adapted to warm climates and which react unfavorably when exposed to low temperatures. However, in each case it is likely that heavier individuals are produced at temperatures which are optimal for the species involved. Because of its temperature reactions, the Japanese beetle belongs to the latter group. This idea is substantiated by the fact that, although the species is largely northern in distribution, the genus *Popillia*, to which it belongs, is predominantly tropical in distribution (Smith and Hadley, 1926).

The constancy in the number of columnar cells observed at both 20° and 25° C. indicates that temperature has little or no effect on cell number in the Japanese beetle. A number of studies have been made on the effect of temperature on the number of eye facets and the bristle number of *Drosophila melanogaster*. Seyster (1919) and Krafka (1919) reported that lower developmental temperatures resulted in a higher facet number in bar-eyed flies, and Luce (1926) found the reverse condition to exist in the case of *infrabar*. Eigenbrodt (1930) observed that the number of circumocular and ocellar hairs of this species varies inversely with temperature. Since each facet of an insect eye indicates the presence of a definite number of cells, and since the hairs are usually developed from single, modified, hypodermal cells, these studies indicate a change in the number of cells of the adult as the developmental temperature is changed. However, the results obtained on the Japanese beetle are not strictly comparable with those on *Drosophila*, since no observations were made on the cell number of adult beetles reared at different temperatures.

The observation that the columnar cells of the mid-intestine are smaller in Japanese beetle larvae grown at lower temperatures is opposed to the observations of many investigators (see Bělehrádek, 1935). Very little exact experimental evidence is available pertaining to the effects of temperature on the cell size of insects. Alpatov (1930) studied the size of the hypodermal cells of the wing of *Drosophila* grown at the temperatures of 18° and 28° C. The hypodermal cells were not measured, but their size was calculated by counting the number of hairs on a definite area of the wing. He found that the larger the wing, the smaller the number of cells on an area of 0.1 mm.², and consequently the larger the individual cells. Alpatov observed that the wings were larger in flies reared at the lower temperature. Hence, the hypodermal cells were also found to be larger at the lower temperature. It seems likely that the size of the insect is definitely correlated with the size of its constituent cells and that, irrespective of temperature, the larger individuals have larger cells.

SUMMARY

1. Japanese beetle larvae reared at 20° were found to be smaller than those reared at 25° C. The average weights of freshly molted second instars at each temperature were 13.1 mg. and 17.5 mg., respectively. The corresponding weights of freshly molted third instars were 49.0 mg. and 79.7 mg.

2. In spite of this difference in weight at the two temperatures, no differences were observed in the number of columnar cells in the mid-intestine of larvae of a given stage.

3. Except in newly hatched larvae, the columnar cells of the mid-intestine are definitely smaller in individuals reared at the lower temperature. Their difference in size is roughly proportional to the difference in weight of the larvae.

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THE RESPIRATORY METABOLISM OF THE NERVES OF THE BLUE CRAB (*CALLINECTES SAPIDUS*)

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IN A recent study on the metabolism of the peripheral nerves of the lobster, Chang (1931) made the interesting observation that there was a difference in the oxygen consumption of the nerves of the claw as compared with the walking legs. Fragmentary data from numerous sources indicates that a similar difference exists in several other forms of Crustacea. The fact that there should be any great difference in the metabolic rate in two nerves of such similar morphological structure is in itself interesting.

In the present paper a study has been made of the oxygen consumption of the peripheral nerves of the blue crab (*Callinectes sapidus*). Data are presented which indicate a marked difference in the metabolic rate of the nerves of the walking leg and claw. Supplementary experiments are also reported which lend support to the belief that this is an intrinsic property of these nerves and not the result of physical factors in experimental procedure.

MATERIAL AND METHODS

The common blue crab, collected in the vicinity of Woods Hole, Massachusetts, was used throughout the study. The animals were collected daily by the staff of the Marine Biological Laboratory. No attempt was made to select the animals of the same size, but the average was about 6 inches across the carapace.

Two methods were employed in removing the nerves from the appendages. The first consisted of carefully dissecting the nerve from its position in the muscles. This proved at times very difficult because of the similarity of color between the nerves and the muscles, and often resulted in injury to the nerve. The second, and probably the most satisfactory, method was to break the appendage at the joint nearest the distal end and carefully strip the nerve from the inside of the remaining segments. As soon as the nerve was obtained, it was placed in several drops of serum, collected from the animal, and was immediately transferred to the respirometer.

The type of respirometer employed was a modified Fenn manometer. All experiments were carried out at a constant temperature in a bath provided for that purpose. After sufficient time had elapsed to insure equilibrium of pressures in the manometer, readings were made every 15 minutes until the experiment was complete. Most of the experiments required from 4 to 6 hours. The nerves were removed from the manometer, placed upon blotting-paper to remove excess moisture, and then immediately weighed. All computations were made in cubic millimeters of oxygen consumed per gram of moist nerve per hour.

¹ I wish to acknowledge aid from the Hendricks Research Fund, Syracuse University.

RESULTS AND DISCUSSION

The first two series of experiments consisted of measurements on the claw nerve at temperatures of 25°8 C. and 20°4 C., respectively. In both series paired nerves from the same animal were tested in separate manometers. In the first series eleven animals were used, and the results are presented in Table 1. Probably the most striking observation to be made from this table is the large variation in the oxygen consumption of the nerves from different animals. On the other hand, paired nerves of the same animal show a mean difference of about 4.5 per cent. Four different respirometers were used more or less indiscriminately throughout the course of the work, so that the large variation could hardly be attributed to faulty calibration.

TABLE 1
OXYGEN CONSUMPTION OF CLAW NERVE AT 25°8 C.

Animal No.*	Manometer No.	Weight in Milligrams	O ₂ Consumed per Gram per Hour	Animal No.*	Manometer No.	Weight in Milligrams	O ₂ Consumed per Gram per Hour
1 { R.....	4	28	127.31	7 { R.....	1	39	186.42
1 { L.....	3	29	130.37	7 { L.....	2	27	185.40
2, R.....	1	29	97.57	8 { R.....	3	24	152.99
3 { R.....	3	36	109.10	8 { L.....	4	25	159.58
3 { L.....	4	43	98.01	9, R.....	1	20	128.56
4, R.....	1	39	126.61	10 { R.....	3	48	122.03
5 { R.....	3	73	116.88	10 { L.....	4	45	132.04
5 { L.....	4	67	121.86	11 { R.....	3	25	128.00
6 { R.....	3	38	112.44	11 { L.....	4	27	145.93
6 { L.....	2	40	112.20	Average	136.40

* "R" indicates right appendage; "L" indicates left appendage.

This series of experiments was terminated when it became evident that a rapid increase in oxygen uptake occurred approximately 4 hours after the experiment was started. This, according to Shaffer, Chang, and Gerard (1935), is probably the result of bacterial action. In an attempt to delay this apparently abnormal increase in oxygen consumption a series was carried out at 20°4 C. The results of this series are found in Table 2. Essentially the same results were obtained in this series as in series I except that the values for oxygen consumption were somewhat lower, which is to be expected in view of the lower temperature. The normal range of activity, however, was not greatly influenced by the lower temperature; and in no case was it possible to get constant readings for more than 5½ hours without a rapid increase in the oxygen uptake.

In the third series of experiments an attempt was made to compare the oxygen consumption of the nerves from the claw appendage and the first walking leg. Nerves from the same side were tested in separate manometers. The data presented in Table 3 show the results of eleven experiments, which are representative of a total of thirty. These data further emphasize the marked difference in the oxygen requirement in different

animals for both walking-appendage and claw nerves. Furthermore, a striking difference exists in all cases between the two kinds of nerves. The average oxygen consumption for the walking-leg nerve was found to be 156.10 cu. mm. per gram weight per hour, as compared to 99.09 for the claw nerve. These findings are not entirely in accord with

TABLE 2
OXYGEN CONSUMPTION OF CLAW NERVE AT 20°4 C.

Animal No.	Manometer No.	Weight in Milligrams	O ₂ Consumed per Gram per Hour	Animal No.	Manometer No.	Weight in Milligrams	O ₂ Consumed per Gram per Hour
1 { R.....	3	41	84.44	7 { R.....	3	40	101.19
1 { L.....	4	45	88.13	7 { L.....	1	39	111.35
2 { R.....	3	31	94.15	8 { R.....	4	35	151.46
2 { L.....	4	35	88.86	8 { L.....	3	34	152.32
3, R.....	2	24	76.37	9 { R.....	2	30	91.90
4 { R.....	3	30	139.62	9 { L.....	1	35	101.92
4 { L.....	4	32	145.65	10 { R.....	4	28	77.28
5 { R.....	3	45	67.77	10 { L.....	1	25	72.27
5 { L.....	2	42	72.27	11, R.....	3	44	102.43
6 { R.....	3	27	125.42	Average.....			104.74
6 { L.....	4	25	150.10				

TABLE 3
OXYGEN CONSUMPTION OF THE CLAW AND WALKING-APPENDAGE NERVE AT 20°4 C.

ANIMAL No.	CUBIC MILLIMETERS OF O ₂ CONSUMED PER GRAM PER HOUR		ANIMAL No.	CUBIC MILLIMETERS OF O ₂ CONSUMED PER GRAM PER HOUR	
	Walking	Claw		Walking	Claw
1.....	173.07	152.32	7.....	166.60	103.00
2.....	128.10	91.90	8.....	122.47	70.47
3.....	192.10	101.92	9.....	117.65	72.54
4.....	167.54	72.27	10.....	145.53	120.15
5.....	157.63	102.43	11.....	271.42	161.22
6.....	77.22	41.95	Average....	156.10	99.09

those of Chang (1931), who showed that the walking-leg nerve of the lobster consumed less oxygen per hour than the claw nerve.

In an attempt to find a possible reason for this large difference in the metabolic rate of different peripheral nerves in crabs, a slight change in experimental procedure was devised. The possibility suggested itself that the claw nerve, being larger in diameter and thus having less surface in comparison to the mass, might allow slower exchange of gases

and therefore a slower oxygen uptake. In several experiments the claw nerve was split so that the diameter was approximately the same as the walking-leg nerves. The structure of the nerve lends itself well to separation, apparently with little injury. The data obtained were, in general, identical with the results obtained for the whole nerve. This, it would seem, tends to eliminate the factor of nerve diameter and indicates an intrinsic metabolic difference in the nerves of the claw and walking leg of the blue crab. This does not appear to be an unreasonable assumption in light of the results obtained by Guttman (1935) and Shapiro (1936), in which they demonstrated a regional difference in the metabolic rate within the same nerve, the optic nerve of the *Limulus*.

SUMMARY AND CONCLUSIONS

1. The oxygen consumption of the peripheral leg nerves of the blue crab (*C. sapidus*) was determined by means of a modified Fenn respirometer.
2. A wide variation in metabolism was obtained between the same nerves in different animals.
3. The oxygen consumption of the claw nerve was found to average 136.40 cu. mm. per gram weight per hour at 25° 8 C. and 104.74 cu. mm. per gram weight at 20° 4 C.
4. The metabolic rate of the walking-leg nerve was found to be about 56 per cent greater than the claw nerve.
5. The diameter of the nerve is apparently not an important factor.

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REACTIONS OF COLPODA DUODENARIA TO ENVIRONMENTAL FACTORS. II. FACTORS INFLUENCING THE FORMATION OF RESTING CYSTS¹

(Four figures)

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THE validity of results in biological experimentation obviously presupposes the reproducibility of the results under adequately standardized conditions. This would further imply that the conditions to be standardized should include the experimental material no less than the experimental methods employed.

Also, from recent studies (Taylor, Brown, and Strickland, 1936) relating factors of the environment to reactivities of *Colpoda duodenaria* which involve its protoplasmic reorganization, it is evident that the stage of this reorganization definitely enters into the requirement for adequate standardization of this experimental material. This is in line with the general assumption that the developmental stage of the cell or organism, as well as its genetic and environmental history, is an indispensable component of the conditions necessary for reproducible results.

And since protoplasmic reorganization occurs in the resting cysts of *C. duodenaria*, whose encystment and excystment we can induce at will, we are afforded the unique prospect of securing and maintaining, perhaps indefinitely, any desired quantity of experimental material of a known developmental stage whose genetic and environmental conditions may be satisfactorily standardized and so critically studied. Accordingly, were this prospect fully realized, we might then properly regard the induced resting cyst of this organism as a standard living cell, which should serve as an essential point of reference for experimental procedure and for reliable interpretation of experimental results. Our investigations, covering several years, on the nature and factors of induced cystment of *C. duodenaria* have been carried out with the foregoing purpose in view. Consequently, the results herein reported have been encouraging.

These results followed our efforts to determine some of the environmental factors which induce the change from a temporary cyst to a resting cyst and the conditions which must be controlled when uniform resting cysts are required. To this end we have investigated the effect (1) of the concentration of the *Colpoda* and (2) of the volume of the medium containing them, on the number of resting cysts induced.

¹ These studies were supported by a grant from the Rockefeller Foundation.

MATERIAL AND METHODS

For these experiments we have used the cultures of *C. duodenaria*, which have been maintained in thriving conditions in these laboratories for 7 years. Since we wished to determine the percentage of resting cysts formed in food-free medium by different concentrations of *Colpoda* in various volumes of medium, it was necessary to eliminate from the cultures, as far as possible, all organisms which would form cysts of diameter greater than $17\ \mu$. Under the conditions of induced encystment, if the resulting cysts are larger than $17\ \mu$, they cannot be relied upon to remain encysted. The larger cysts usually divide and excyst, producing two small daughters. Under certain conditions (unfinished studies), mainly of high concentration, cysts of greater than $17\ \mu$ diameter can be induced to remain encysted, i.e., to form resting cysts; but for these experiments such sizes were, as far as possible, eliminated.

Colpoda of the required size were produced by controlling the supply of food, the aim being to cover the bottom of the dish with large numbers of quadrigenic division cysts of diameters about 28 or 30 μ . The daughters are then of the right size for these experiments.

Colpoda in sufficiently food-free medium were obtained by two methods, both of them quite satisfactory. When small numbers were required, the ciliates were transferred with sterile micropipettes through three or more dishes of sterile medium. When greater numbers were needed, the culture was washed three or more times in the centrifuge. It is claimed not that these organisms were sterile but that they were so free from bacteria that in a nonnutritive medium, such as was used in this work, the effect of food was negligible.

In the first series the volume of medium, called, for convenience, the "drop," was $\frac{1}{4}$ cc., and the concentrations of *Colpoda* ranged from zero to 4,000 per cubic centimeter. For concentrations up to 500 per cubic centimeter the required number of protozoa was picked up in a pipette calibrated to $\frac{1}{4}$ cc. For higher concentrations the washed culture was diluted approximately to the concentration required, and a drop of $\frac{1}{4}$ cc. was picked up in a pipette and transferred to a Columbia dish for observation. Since food was absent, the *Colpoda* all encysted; when encystment was complete, the cysts were counted. In the few cases in which the number of cysts was too great to be counted accurately, separate cultures of cysts were counted with care, and thereafter the total number was estimated.

Soon after encystment was complete, excystment, if any, began. Unpublished studies show that, in the absence of food, these excysted organisms re-encyst and that some of them may form resting cysts. This re-encystment takes place in a different environment from the first. We are concerned in this paper only with the phenomena of the first encystment, and so at intervals the excysted *Colpoda* were removed with an extremely small pipette. Twenty-four hours after encystment the number of resting cysts was counted. This number, added to the number of ciliates removed, gives a useful check on the total of organisms originally in the drop.

The same experiment was repeated with drops of $1/40$ cc. In this case it was found advisable to put the drops on the cover of a small Petri dish. Evaporation was hindered by additional moisture in the dish.

The same experiment was repeated with drops of $1/400$ cc. In this case each drop was placed on a cover slip, which was reversed over a deep hollow-ground slide containing

sufficient moisture to saturate the atmosphere. The cover slip was sealed onto the slide with water, which was replenished when necessary. Encystment was usually complete after 3 hours; and excystment, if any, after 6 hours. The cover slips were then immersed on a rack in a large volume of sterile balanced salt solution. The following morning only the resting cysts remained on the cover slips.

The same technique was employed for drops of $1/4,000$ cc., except that usually 4 drops were put on each cover slip. When more than 1 drop was put on a cover slip, the outline of each drop was marked with India ink. Drops of $1/40,000$ cc. were much more difficult to prepare. However, with practice and a suitable point on a micropipette calibrated to $1/4,000$ cc., it was possible to put onto a cover slip one-tenth of $1/4,000$ cc. in 10 drops of approximately equal size. Drops obviously too large or too small were discarded.

All experiments were carried out in a constant-temperature room at 20° C. The relative humidity was normally maintained at 65 per cent; but in order to reduce evaporation, it was raised to 80 per cent and more while the small drops were being prepared.

To study the formation of resting cysts in drops much smaller than $1/40,000$ cc., a micromanipulator with injection apparatus (Taylor, 1925) was used. To it was attached a capillary tube of soft glass, into which was drawn a drop of food-free medium containing one or more *Colpoda*. On each side of the drop, but separated from it by a small air space, was another drop of medium to prevent evaporation. The piece of the capillary containing the drops was then broken off, and the two ends were plugged with soft paraffin. The small section of capillary was then affixed to a cover slip with wax; the cover slip was reversed onto a hollow-ground slide for convenience in handling.

EXPERIMENTAL

By the methods just described, 67 drops of $\frac{1}{4}$ cc. each were tested to determine the percentage of resting cysts formed at various protozoan concentrations. When this percentage of resting cysts is plotted on linear paper against the concentration of ciliates, the curve is apparently logarithmic. Figure 1 shows the curve for $1/4,000$ cc. The curves for $\frac{1}{4}$ cc. and all other volumes are similar. This is confirmed by plotting the percentage of resting cysts against the concentration on a logarithmic scale. For this purpose the 67 cases in which the drop was $\frac{1}{4}$ cc. were divided into five groups. In each group the percentage was determined from the mean number of resting cysts formed and the mean concentration of protozoa. The curve (Fig. 2) is then a straight line, which cuts the axis of the abscissas at about 16 organisms per cubic centimeter and the 100 per cent line at about 6,500 per cubic centimeter. From this it appears that, when 1,625 *C. duodenaria*, i.e., 6,500 per cubic centimeter, in sizes too small to result in binary fission after encystment, are present in $\frac{1}{4}$ cc. of food-free balanced salt medium, they will all form resting cysts. When 4 such (or less) are present in the same amount of medium, no resting cysts will be formed, but the cysts induced by the absence of food will all reorganize and excyst.

To determine the curve for drops of $1/40$ cc. we have 138 cases, divided into seven groups. When the mean percentage of resting cysts is plotted against the logarithm of the mean concentration in each group on a logarithmic scale, we have seven points, which appear to lie on a straight line parallel to the $\frac{1}{4}$ cc. line (see Fig. 2). We see that no resting cysts are formed unless the concentration is 225 or more per cubic centimeter,

i.e., 5.6 organisms in the drop, and that it requires a concentration of 105,000 per cubic centimeter, i.e., 2,625 per drop, to induce permanent encystment in all of them.

For drops of $1/400$ cc. we have 279 cases, divided into nine groups. The concentration must be 850 per cubic centimeter, i.e., 2.1 per drop, before any resting cyst is formed, and 410,000 per cubic centimeter to induce 100 per cent permanent encystment.

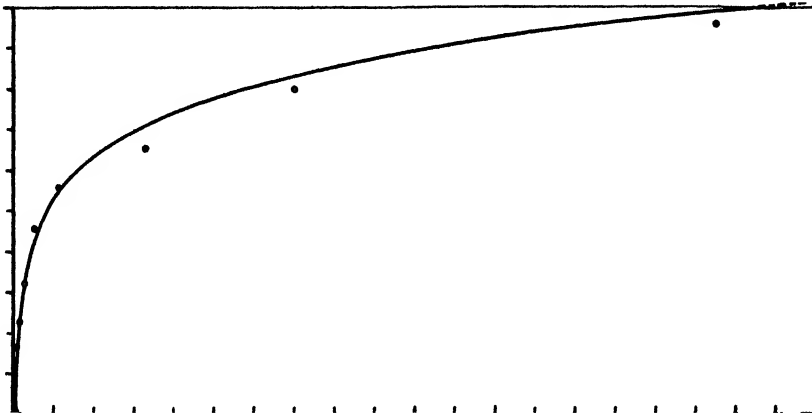


FIG. 1.—Induced resting cysts: percentage of concentration in $1/4,000$ cc. of medium

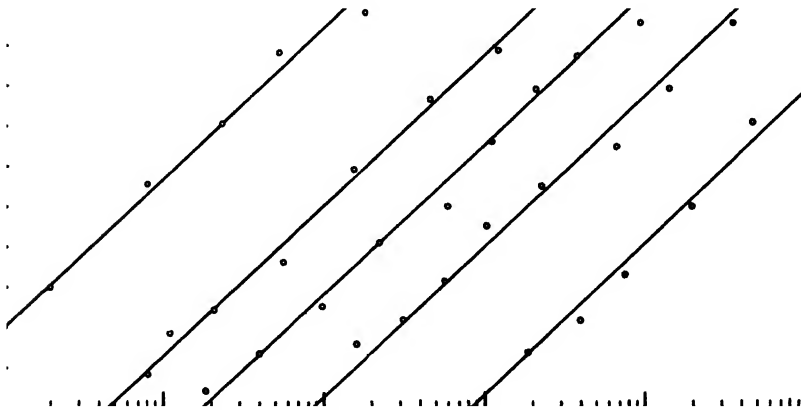


FIG. 2.—Resting cysts: percentage plotted against log concentration of protozoa in various volumes of medium.

The curve is also a straight line parallel to the former two. Similarly, for $1/4,000$ cc. we have 466 cases in nine groups. One organism per drop, i.e., 4,000 per cubic centimeter (34 cases), never forms a resting cyst, and a concentration of 1,950,000 per cubic centimeter is necessary for 100 per cent permanent encystment. For drops of $1/40,000$ cc. we have 169 cases in seven groups. No resting cysts are formed at a concentration of 40,000 per cubic centimeter, and for 100 per cent permanent encystment a concentration of 17,200,000 per cubic centimeter is necessary.

The curves for all these drops of different volumes appear to be parallel straight lines on semilogarithmic paper, thus showing that the smaller the drop the greater the concentration of protozoa necessary to induce formation of resting cysts.

It will be sufficient to give (Table 1) the actual figures and calculations for any one of these curves, e.g., for 1/4,000 cc., as the others are similar, and the points can be seen in Figure 2.

TABLE 1
(Size of drops, 1/4,000 cc.)

Concentration of <i>Colpoda</i> per Cubic Centimeter	Number of Cases	Mean Con- centration	Mean Percent- age of Resting Cysts	Standard Deviation σ	Standard Error*
4,000.....	34	4,000	0	0	0
8,000.....	104	8,000	16.4	22.45	2.21
12,000-20,000.....	107	15,800	22.5	27.8	2.07
24,000-36,000.....	41	28,400	32.0	27.3	4.26
40,000-60,000.....	38	57,100	45.5	22.75	3.68
64,000-236,000.....	71	112,800	55.5	19.5	2.32
240,000-396,000.....	19	331,000	65.2	14.6	3.34
400,000-1,196,000.....	38	700,000	79.5	16.45	2.66
1,200,000 and over.....	14	1,753,000	95.7	5.47	1.46

* The mean standard error of the mean is ± 3.32 for $\frac{1}{4}$ cc., 3.59 for 1/40 cc., 3.07 for 1/400 cc., 2.50 for 1/4,000 cc., and 3.72 for 1/40,000 cc.

In Figure 3 we have plotted the minimum protozoan concentration (taken from Fig. 2) necessary to induce 100 per cent formation of resting cysts in each size of drop. The curve resembles a hyperbola whose asymptotes are nearly the co-ordinate axes. This shows clearly that, as the volume of medium decreases, the concentration of organisms necessary to induce 100 per cent formation of resting cysts increases very rapidly. We have omitted from Figure 3 the volumes $\frac{1}{4}$ cc. and 1/40,000 cc. because, to include them, it would have been necessary to reduce the units of both axes tenfold.

The question now arises: Can one *C. duodenaria* so condition its own environment that it will itself form a resting cyst? From Figure 3 it appears that this might occur if the drop were sufficiently small. If the drop is extremely small, the concentration required to induce 100 per cent formation of resting cysts should be correspondingly enormous. When a minute drop containing one or more *Colpoda* is isolated in a capillary tube, it is possible to obtain very high concentration in very small volumes of medium. We have 100 cases for consideration, in which the drops ranged from 5×10^{-7} cc. upward. In no case did one *Colpoda* in a drop form a resting cyst. As the number of protozoa increased up to 40, the percentage of resting cysts increased approximately along a logarithmic curve. It was not possible, owing to technical difficulties, to increase the number of ciliates or to diminish the volume of medium sufficiently to obtain 100 per cent formation of resting cysts; or at least the time required to do so seemed incommensurate with the value of the expected results.

Figure 3 also suggests that one *Colpoda* in a drop might induce itself to form a resting cyst if the volume of medium were sufficiently large. This is supported by Figure 4, which illustrates the actual number of organisms necessary to induce 100 per cent of resting cysts in drops of different sizes.

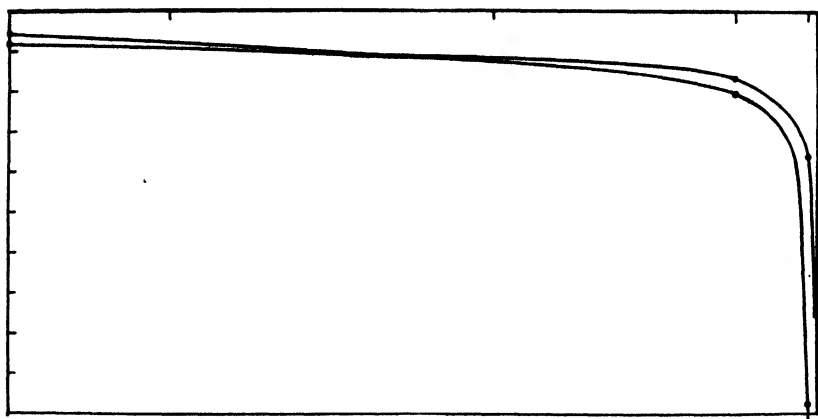


FIG. 3.—Graphs indicating possible relationship between induction of resting cysts and the surface : volume ratio of the drops. (a) Minimum concentration to induce 100 per cent of resting cysts plotted against volume of the drop (○); (b) surface-volume ratio (air-water surface) plotted against volume of the drop (●).

Vol.	Surface-Volume Ratio	Concentration
1/4.....	7.24	6,500
1/40.....	15.5	105,000
1/400.....	33.6	410,000
1/4,000.....	72.4	1,950,000
1/40,000.....	155.0	14,200,000

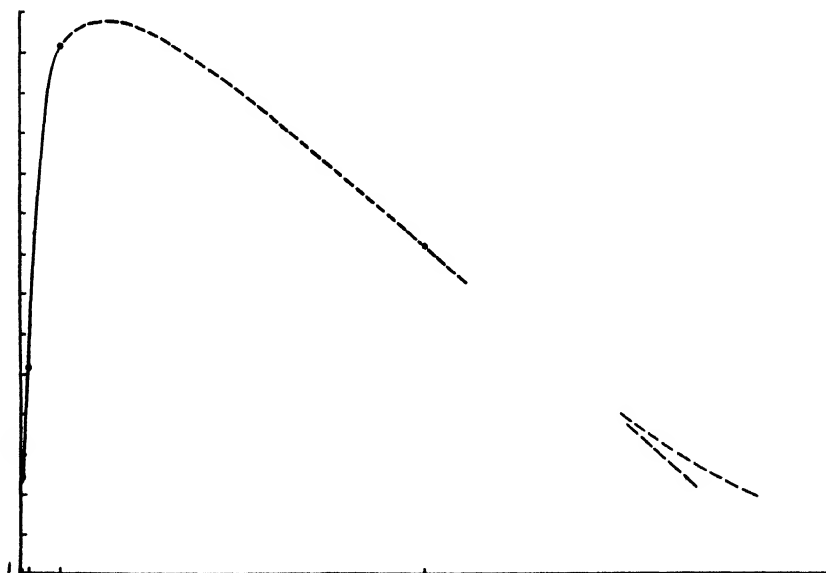


FIG. 4.—Number of *Colpoda* per drop necessary to induce 100 per cent of resting cysts in drops of various volumes.

We have not sufficient data to extrapolate this curve beyond $\frac{1}{4}$ cc.; but in order to find out if it cuts the axis of the abscissas in the neighborhood of 1 cc., we made thirteen tests of one *Colpoda* in $\frac{1}{4}$ cc. and eight tests of one organism in 1 cc. In no case was a resting cyst formed. Since it is very difficult to find a small cyst in such large volumes of medium, these tests were discontinued for the present.

In Figure 3 we have also plotted the surface-volume ratio (i.e., the air-water surface) for the different volumes of medium, assuming, therefore, that the drop is a segment of a sphere and that the height of the segment is one-third of the radius of the sphere. The scale of ordinates for this curve is on the right side of the figure. The striking similarity between the two curves in Figure 3 will be discussed later.

DISCUSSION

As previously shown (Taylor and Strickland, 1938), when food is removed from their medium, all *C. duodenaria* will encyst. If large enough, they will ordinarily then undergo fission and emerge from the cyst as two, four, or even eight daughters, according to size of the cyst. Smaller *Colpoda*, forming cysts of diameter less than about $17\ \mu$, do not divide; but, depending upon conditions described in foregoing paragraphs, they may either (1) redifferentiate in due course and emerge as normal free-swimming forms or (2) form resting cysts which are readily identifiable, owing to changes in their appearance and to a perceptible diminution in size. The latter will remain dormant until redifferentiation is initiated by a suitable stimulus.

The experimental data herein presented have had to do primarily with resting cysts. For purposes of comparison throughout this discussion, however, it will be well to bear in mind also the other type (see [1] above) of nondivision cysts, which are only temporary. Following emergence, if no food is available, the free-swimming forms again encyst. This cycle is repeated again and again at fairly regular intervals, with a diminishing size of the cyst.

Our experimental results recorded above indicate that there may be no intrinsic difference between those *C. duodenaria* that will form these temporary cysts, on the one hand, and those that will form resting cysts, on the other. Which of these two types of cysts will be formed may depend rather on the conditioning of the food-free medium at the time encystment takes place, for in a given volume of this medium the percentage of resting cysts formed depends on the number of organisms present, i.e., the concentration of *C. duodenaria* per cubic centimeter of the food-free medium.

This significant finding is well demonstrated in the curve of Figure 1, as plotted on linear paper for various concentrations of *Colpoda* in drops of $\frac{1}{4},000$ cc. of balanced salt medium. In all concentrations below 4,400 per cubic centimeter redifferentiation and emergence ensue, while in all higher concentrations up to 1,950,000 per cubic centimeter the percentage of resting cysts formed increases with increasing concentration, at first rapidly and then more and more slowly, up to 100 per cent of resting cysts. In concentrations above 1,950,000 per cubic centimeter there are, of course, 100 per cent of resting cysts; but the extrapolated continuation of the curve may indicate that such concentrations produce a surplus of some unknown factor or factors, which theoretically, could induce the formation of more resting cysts than there are organisms in the drop.

Certain technical difficulties have thus far not been fully overcome in the transfer of the organisms to a balanced salt, food-free medium. For each test the culture of *Colpoda* has to be washed as free as possible of bacteria and of organic solutes (Taylor and

Strickland, 1938) by repeated use of the centrifuge. Moreover, there are commonly present a few *Colpoda* large enough to form digenic division cysts, whose emerged progeny thus count doubly as free-swimming forms and reduce the percentage of resting cysts.

To these and other technical difficulties may be ascribed the relatively high values of the standard deviation of the mean (Table 1). This is obvious especially in tests where a small change in the concentration produces a large change in the percentage of resting cysts (cf. steep portion of curve in Fig. 1) or where the few organisms in a small drop (1/4,000 cc.) represent in the calculation a large number per cubic centimeter. For example, two organisms in such a drop correspond to a concentration of 8,000 organisms per cubic centimeter; and since the possible results are 0 per cent, 50 per cent, or 100 per cent of resting cysts, a large standard deviation can be expected. The maximum value of σ for these tests is ± 27.8 per cent; but as the number of organisms in the drop increases, σ falls progressively to ± 5.47 per cent. Owing to the large number of cases in each group of tests, the standard errors of the mean are reasonably small.

It is evident from Figure 1 that, for a given volume of food-free medium, the percentage of resting cysts formed is some function of the concentration of the organisms. In Figure 2, however, the graphs show that in drops whose sizes vary by a factor of one-tenth, from $\frac{1}{4}$ cc. to 1/40,000 cc., the ratio values of the percentage of resting cysts to concentration of organisms vary with the size of the drops. On semilogarithmic paper the graphs are all straight lines and parallel. These data indicate that the surface of the drop, as well as its volume, is a limiting factor, since the smaller the drop the greater is the concentration of organisms required to induce the formation of a given percentage of resting cysts.

Further demonstration of this relation of the size of the drop of food-free medium to the concentration of organisms in the induced formation of resting cysts of *C. duodenaria* is given in the curves of Figure 3. In the first of these, marked *a*, the concentration of *Colpoda* (in millions per cubic centimeter) necessary for the induction of 100 per cent resting cysts is plotted on linear paper against the volume of the drops. The resulting hyperbolic curve is strikingly similar to the second curve, marked *b*, of the surface-volume ratio plotted also against the volume of the drops. Curve *a* on logarithmic paper gives approximately a straight line, so that it is nearly a rectangular hyperbola. The curves *a* and *b* are, however, so nearly alike that it seems a plausible hypothesis that the surface-volume ratio is indirectly a controlling factor in the induction of resting cysts. If the surface-volume ratio is plotted on logarithmic paper against the corresponding concentration of *Colpoda* necessary to induce 100 per cent of resting cysts, the five points obtained are also approximately on a straight line.

Extrapolation of the arms of curve *a* in Figure 3 might suggest the possibility of preparing, on the one hand, a drop small enough (cf. vertical arm) or, on the other hand, a drop just large enough (cf. horizontal arm) so that in either case the limiting factor might enable one *Colpoda* so to condition the drop that the organism would be induced to form in it a resting cyst. Since the volume of the cyst would be about $2,500 \mu^3$, or 2.5×10^{-9} cc., and since for inducing resting cysts the required concentration of *Colpoda* increases nearly tenfold when the volume of the drop is decreased from 1/4,000 cc. to 1/40,000 cc., then by extrapolation it would appear that the volume of the theoretical drop in which one *Colpoda* would induce itself to form a resting cyst might be smaller than the organism itself.

An extrapolation of the horizontal arm of curve *a* in Figure 3 is indicated by the dotted line in Figure 4. Assuming that this arm very closely approximates the axis somewhere between $\frac{1}{4}$ cc. and 1 cc. and that, accordingly, one *Colpoda* might induce itself to form a resting cyst in a drop of $\frac{1}{2}$ cc., or in a drop of 1 cc., thirteen tests were carried out with one organism in $\frac{1}{2}$ cc., and eight tests with one organism in 1 cc. In all tests the results were apparently negative, although the difficulties of following the fate of an organism so small in such large drops are obvious.

It is, of course, quite possible that in either or both of these extreme parts of curve *a* in Figure 3, the surface-volume factor for induced resting cysts may be modified by some other unknown factor or factors so that the curve might thereafter tend to become parallel to the co-ordinate axes. In such cases one could then expect that a minimum constant number of *Colpoda* would be necessary to produce 100 per cent of resting cysts and that this number might in either case be greater than one.

The experimental results reported in this paper and analyzed by graphs in the several figures appear, therefore, to establish a relation between the surface-volume ratio of the food-free drops containing *C. duodenaria* and the induced formation of its resting cysts. Just what specifically is the essential factor or factors involved in this relation between the environmental medium and the reacting organism is at present not clear.

Several possibilities are fairly obvious. Since to induce resting cysts the concentration of organisms must be increased as the size of the drop is decreased, i.e., as the surface-volume ratio of the drop increases, one might at once suppose that some essential volatile substance, such as CO₂, is escaping into the gas phase, or that the O₂ diffusing from the gas phase into the drop may counteract the environmental factor(s) that induce resting cyst formation. Thereby, in either case, the food-free medium becomes modified at a rate varying approximately with the surface-volume ratio of the drop. And since this modification of the medium would presumably tend to inhibit the formation of resting cysts, then, in order to offset this modification, the concentration of *Colpoda* would have to be increased as the surface-volume ratio of the drops is increased, i.e., as the drops are made smaller and smaller.

This could, of course, presuppose that the organisms are adding some "r-substance" or substances to the medium which, at threshold concentrations, condition the medium and, in turn, induce the formation of resting cysts. Carbon dioxide or some unoxidized metabolite(s) might be the r-substance(s) in question. In any case, its concentration would evidently be affected not only by the rate of its production (hence by the relative number of organisms present) but also, for small drops, by its rate of escape through the surface of the drop, or, if surface active, by its accumulation at the drop's surface, or by its change through oxidation or otherwise to a biologically inactive state. For the large drops, however, as represented by the horizontal arm of curve *a* in Figure 3, the threshold concentration of this hypothetical substance, at a given rate of its production, might depend chiefly on its diffusion, since the surface-volume ratio should here become less and less a limiting factor.

These considerations obviously relate to the possible origin, nature, and identity of the factor or factors that activate *Colpoda* to form resting cysts. Investigations now under way may provide these essential data on the activating agent(s) thus operative. One may well bear in mind, however, that it is the reacting protoplasm, rather than activating agents, which primarily concerns the biologist. Clearly enough, for example, *Colpoda* may form a resting cyst instead of a temporary cyst, previously referred to,

upon reacting to its conditioned medium, so that the former cyst must differ from the latter, owing to some change in its protoplasmic state. This state may then, at will, be reversed upon the addition of a suitable excystment medium.²

Some things are known about the cystment process in *Colpoda* to which brief reference may here be made as a possible means of better understanding this essential difference between its resting cyst and its temporary cyst. A detailed study has been made (Taylor and Garnjobst, 1939) of protoplasmic reorganization in *C. duodenaria* during fission. Here all protoplasmic organelles, such as cilia, and the fibrillar system, are re-sorbed, and new organelles are redifferentiated in the resulting daughters. No detailed study of structural changes during its formation of resting cysts has, as yet, been made; but results thus far obtained make it appear altogether probable that reorganization then also occurs, as during fission. Assuming this to be so, its possible significance will be referred to farther on.

From studies on the effects of X-rays on normal excystment time of *Colpoda* (Taylor, Brown, and Strickland, 1936) it was found that for a given dose of 38,400 *r*-units applied at various times during the first hour of experimentally induced excystment, the excystment time was increased in each case approximately threefold. But the same dose applied at intervals after the first hour was less and less effective, so that the excystment time for organisms X-rayed near their emergence was approximately normal. Evidently, then, the X-ray effect varied with the stage of excystment process when the dose was applied.

It was further shown (Brown and Taylor, 1938) that during the first 20–25 minutes of induced excystment, the excystment time was dependent upon the concentration of the excystment medium but independent of the O₂ tension. For the remainder of the first hour, however, the excystment time varied with the O₂ tension but was independent of the concentration of the excystment medium. This would indicate that at the beginning of induced excystment some essential ingredient of the excystment medium was diffusing into the protoplasm with which it combined, thus to initiate the excystment processes requiring oxygen. If, as we assumed, this combination of diffused ingredient and protoplasm involved enzyme synthesis, then we could further suppose that the essential ingredient added from the excystment medium was conceivably a co-enzyme (prosthetic group), which combined with a protein constituent of the protoplasm, thereby forming the required enzyme for the initiation of processes leading to visible protoplasmic redifferentiation and excystment.

Could these events initiating redifferentiation and excystment be thus reasonably postulated, then it would seem to follow that the formation of a resting cyst may involve in its dedifferentiation an inactivation of certain enzyme(s) whose degradation was brought about by a threshold concentration of the hypothetical *r*-substance(s) in the conditioned medium. This might imply, let us suppose, a splitting-off and loss, from the protein component of such enzymes, of the complementary and simpler prosthetic component which, before excystment will occur, must ordinarily be contributed to the resting cyst from a suitable excystment medium. Temporary cysts, on the other hand, may not, in the absence of a sufficiently conditioned medium, suffer this degradation of the enzymes that activate redifferentiation and excystment, so that excystment in these temporary cysts ensues spontaneously.

² Haagen-Smit and Thimann recently found (1938) that the potassium or sodium salts of several organic acids were effective agents for excystment of *C. cucullus*.

This hypothesis would seem to agree thus far very well with the experimental data herein presented and previously reported on induced cystment in *C. duodenaria*, and it should provide a satisfactory working basis for our further studies along this line which are now in progress.

Finally, brief mention might be made of the bearing our experimental results may have on those published by several others who have studied cystment in this and other ciliates:

Penn (1934) agrees that crowding induces encystment (in *Pleurotricha lanceolata*) but adds that the effect is due to accumulation of waste products. We have shown (Taylor and Strickland, 1938, Expt. II) that, for *C. duodenaria*, at least, crowding does not induce encystment when sufficient food is present in the medium, and we have further shown (*ibid.*, Expt. V) that a 1 per cent solution of sterile yeast extract can provide sufficient food to maintain the free-swimming state for a comparatively long time. If these observations hold good for *Pleurotricha*, then the three cases in Penn's Table II are open to a different interpretation, namely, (a) 1 animal in 1 ml. of sterile rye extract gave only 1.03 per cent of cysts, as, indeed, would be expected if the rye extract were supplying some nourishment; (b) 10 animals in 1 ml. of rye extract gave 39.33 per cent cysts, as we might expect, since 10 animals would use up the available food ten times as quickly as 1 animal; (c) 1 animal in 1 ml. of rye extract plus excretion products could also be expected to give a high percentage of encystment, because the 10 *Pleurotricha* left for 24 hours in the extract to accumulate excretion products would have used up much of the available food.

Barker and Taylor (1931) state that crowding is of primary importance for the encystment of *Colpoda*; our data confirm this statement, with the reservation that the medium must be free of food. If, as we believe, the protozoa used in their work were the progenitors of the one we are now using under the name of *C. duodenaria* (Taylor and Furgason, 1938), then, in the light of more recent investigations, Table V in their paper seems to offer insufficient data on which to base the foregoing conclusion. In the first 5 cases (abundant bacteria) only a varying number of division cysts, but no permanent cysts, should have been formed. In the next 3 cases (no bacteria) the first has such a low concentration that the protozoa were probably undergoing the rhythmic encystment and excystment (Taylor and Strickland, 1938, p. 401), ending in death from depletion about the fifth day; the second has sufficient concentration, if in food-free medium, to give 50 per cent of permanent cysts in about 5 hours, so probably there was some food available; the third (3,600 per cubic centimeter) in food-free medium should make about 90 per cent permanent cysts within 4 or 5 hours.

Although our data offer no definite evidence of any inhibiting factor other than the presence of food, it is interesting to note that Beers (1927) found for *Didinium nasutum* an inhibition of encystment when the excretion products of *Paramecium* were present in the medium. It is; however, not clear whether there may not have been in the medium sufficient nutriment, such as organic solutes, living or dead bacteria, and other particles, to maintain the free-swimming state and prevent encystment for some time. In Part I of this series we have shown that the metabolites of *Colpoda* and *Pseudomonas* accumulating for 16 weeks do not seem to have any effect on encystment.

Mast and Ibara (1923) state: "Rapid and extensive reproduction of *Didinia* confined to a small space seems to favor encystment and it may be that this is owing to accumulation of waste products." Unless there is at all times sufficient food in the medium

to maintain the culture, we may suppose that crowding of *Didinium* when the food is used up induces encystment, as in *Colpoda*. Their tentative explanation would then be open to question.

SUMMARY

This study of the conditions which induce the formation of resting cysts of *C. duodenaria* reveals that:

1. The percentage of resting cysts formed in a given volume of food-free medium is proportional to the logarithm of the protozoan concentration.
2. The smaller the volume of medium, the greater must be the concentration of protozoa to induce all of them to form resting cysts.
3. For drops of medium from $\frac{1}{4}$ cc. down to 1/40,000 cc. the curve illustrating the variation of this concentration with the volume of the medium resembles a hyperbola.
4. There is a striking resemblance between the curve of concentrations and a curve giving the surface-volume ratio for the various volumes of medium.
5. It is theoretically possible for one *Colpoda* to induce itself to form a resting cyst in extremely small or in large volumes of food-free medium.
6. The possibility of an encystment-promoting factor is briefly discussed.

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CHEMICAL MEDIATION IN CRUSTACEANS. II. THE ACTION OF ACETYLCHOLINE AND ADRENALIN ON THE ISOLATED HEART OF PANULIRUS ARGUS

(Five figures)

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THE presence of relatively large amounts of acetylcholine (Welsh, 1938 and 1939) and choline esterase (Bacq and Nachmansohn, 1937; Marnay and Nachmansohn, 1937) in nervous tissues of decapod crustaceans suggests that chemical mediation of nervous impulses by acetylcholine may occur in this group of invertebrates as it does in the vertebrates. Attempts, thus far, to demonstrate an effect of acetylcholine at the neuromuscular junction of skeletal muscle of crustaceans have not been conclusive (e.g., Katz, 1936), but it has been shown to act on the isolated hearts of *Maia* and *Carcinus* in concentrations of physiological significance (Welsh, 1939). The action on the crab's heart is the opposite of that on the vertebrate heart, since acetylcholine excites the former and inhibits the latter type of heart. Also of interest are the opposed actions of acetylcholine and adrenalin on the vertebrate heart and the excitatory action of both of these substances on the crab's heart.

MacLean and Beznák (1933) had earlier studied the action of acetylcholine and adrenalin on the heart of the crayfish and had extracted a substance having the properties of a choline ester from this heart; but this work was unknown to the writer when the work on *Maia* and *Carcinus* was being done, and not until the present study had been completed did the paper come to my attention. Without knowledge of the work of MacLean and Beznák it seemed desirable to determine the action of acetylcholine and adrenalin on the heart of a representative of the Macrura and especially to obtain more exact quantitative data with a view to extending our knowledge of the mode of action of these two common mediator substances. Certain difficulties were encountered in dealing with the heart chosen for this study; and it was found impossible, in the limited time, to obtain all the desired data on the separate and combined actions of acetylcholine and adrenalin. It was possible, however, to demonstrate that the actions of these two substances, although excitatory, were not identical, and to obtain some idea of the effects of various concentrations. An idea of the arrangement and action of the local nervous system of the decapod heart was also obtained, this being necessary in any detailed analysis of the control of beat of this type of heart.

Certain of the more important findings form the basis of the present paper. A grant from the Milton Fund of Harvard University made this work possible.

MATERIALS AND METHODS

The heart of *Panulirus argus* (Latreille), the sea crawfish, or spiny lobster, of the region of the West Indies, was chosen for the present study. The large size of this heart was not found to be as much of an advantage in the perfusion studies as had been expected,

but it was ideal for determining the arrangement of the ganglion cells in the heart of a marine decapod.

To prepare the heart for perfusion, it was first exposed by removal of a portion of the carapace dorsal to the heart. Preliminary to this, the legs had been cut off and the animal allowed to bleed. Flushing out the pericardial space with perfusion fluid prevented the coagulation of any remaining blood. An incision in the dorsal abdominal artery permitted the insertion of a cannula directly into the cavity of the heart. The ligature, which secured this cannula, was so placed that it also included the sternal artery. This prevented the rapid escape of fluids by way of this blood vessel and helped in maintaining a slight pressure within the heart, which is quite necessary for its normal functioning when isolated. A second thread tied off the ophthalmic and antennary blood vessels and was used to attach the heart to the writing-lever. The ligaments of the heart were then cut, and it was placed in a perfusion bath and the cannula connected, by a two-way stopcock, to a large perfusion bottle and a perfusion tube. By means of the latter, small amounts of test fluids could be introduced to the heart. The rates of flow from these two were adjusted by keeping them at appropriate levels above the heart and by adjustable clamps placed on the rubber connectives leading to the stopcock. An illustration of such a setup may be seen in the first paper of this series (Welsh, 1939).

Since an analysis of the ionic composition of the blood of *Panulirus* was lacking, a perfusion fluid having the composition of that used for *Carcinus*, the green crab, was tried. This contained 100 parts of 0.6 M NaCl, 2.5 parts of 0.6 M KCl, 4.0 parts of 0.4 M $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 7.0 parts of 0.4 M MgCl_2 , and was brought to pH 7.0 with NaHCO_3 . On the first few hearts this seemed to be adequate, as it maintained a regular beat for periods of 10 or more hours. During later work, however, it was obvious that it was not an entirely appropriate mixture. Changes in the potassium and magnesium were tried, and a reduction to 2.5 parts of 0.4 M MgCl_2 helped most in preserving a regular beat over a period of time. Future work on the heart of *Panulirus* should be preceded by an analysis of its blood and the development of a more satisfactory perfusion medium. Stock solutions of atropine sulphate, eserine sulphate, acetylcholine chloride, and adrenalin chloride were made at frequent intervals, usually daily; and appropriate dilutions with the perfusion medium were made just before using. The stock solution of acetylcholine was kept at a pH of 4.0.

The arrangement of ganglion cells and fibers in the heart was demonstrated by staining with methylene blue. The heart was split lengthwise along the ventral side, pinned out flat, and covered with perfusion fluid made slightly acid with HCl (pH 4.0–5.0). To this was added 0.5 per cent methylene blue in the proportion of 15–20 drops of methylene blue to 100 cc. of perfusion fluid. The preparation was placed in a refrigerator for 8–12 hours and then examined. Excellent results were obtained, and it is believed that this was partly due to keeping the preparation at a low temperature.

THE ACTION OF ESERINE, ATROPINE, AND ACETYLCHOLINE

If the local nervous system of the heart of *Panulirus* is cholinergic, as seems to be the case in *Maia*, *Carcinus*, and the crayfish, it should be possible to demonstrate this by the appropriate use of eserine, atropine, and acetylcholine. Eserine is known to potentiate the action of acetylcholine by preventing its hydrolysis by choline esterase. Atropine abolishes the action of acetylcholine when it has a so-called 'muscarin effect'; and the

evidence from *Carcinus* indicated that this, rather than the "nicotine effect," characterizes the action of acetylcholine in the decapod heart.

When the isolated heart of *Panulirus* was perfused with eserine 10^{-5} , two effects were observed, depending on the previous state of the heart. If the heart had been beating in a somewhat irregular manner (e.g., exhibiting a series of grouped beats), a regular beat



FIG. 1.—Record of the effect of perfusion with acetylcholine 10^{-6} between first and second arrows, and then with atropine 10^{-5} , followed by normal perfusion medium. Time interval = 5 seconds in this and subsequent records

FIG. 2.—Showing the effect of acetylcholine 10^{-5} on the same heart from which record in Figure 1 was obtained.

would be completely restored. If the heart had been beating in a regular manner, an observable increase in frequency occurred. After a period of perfusion with eserinated perfusion fluid, followed by a period of washing, a second trial with eserine had little effect on the heart.

Acetylcholine 10^{-10} to 10^{-9} , which were the lowest concentrations used, had effects similar to those produced by eserine, namely, the restoration of a normal beat or a slight

increase in frequency. Acetylcholine 10^{-8} , after eserinizaton, caused an increase in frequency of beat and a slight increase in tonus¹ of certain hearts, while acetylcholine 10^{-7} caused a marked increase in frequency and a decrease in amplitude. Further increases in the concentration of acetylcholine produced further changes, ending in complete systolic stoppage with a concentration of 10^{-4} , from which the heart would not recover until washed for several minutes. A series of records of the effects of high concentrations on the same heart are shown in Figures 1-3. Obviously, such effects as these produced by high concentrations of acetylcholine are not "normal," but they are chosen to illustrate the essential differences in the action of acetylcholine and adrenalin on this heart, which was one of the purposes of this work.

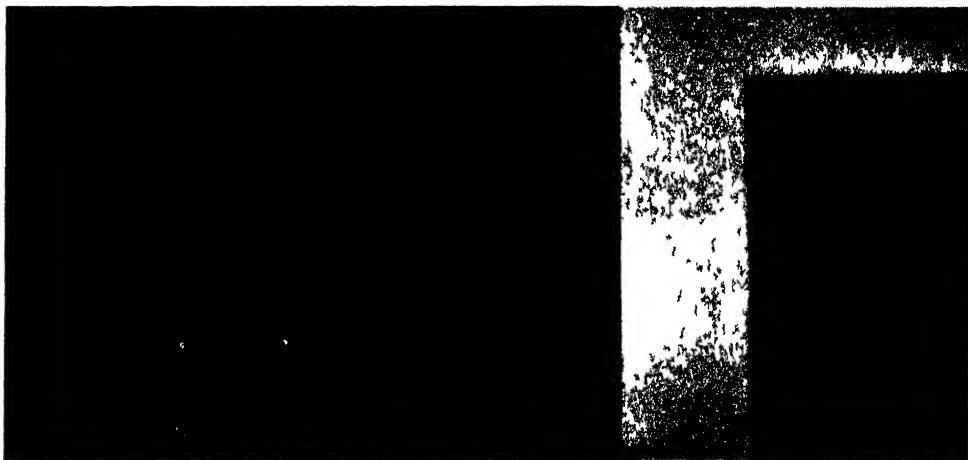


FIG 3 —Showing the effect of acetylcholine 10^{-4} on same heart as Figures 1 and 2. A period of 6 minutes separates the two portions of the record.

Atropine reduces the rate of beat or stops the heart of *Panulirus*, the effect depending on the concentration. In Figure 1 may be seen a record of the effect of atropine 10^{-5} following a period of perfusion with acetylcholine. If the heart is perfused with atropine 10^{-5} for a few minutes and then perfused with acetylcholine, the stimulating action of the acetylcholine is largely abolished, as in *Carcinus*.

THE ACTION OF ADRENALIN

Adrenalin had been found by Bain (1929) to accelerate the heart of *Carcinus* in a concentration of 1:10,000,000. No attempt was made to find the limit of sensitivity of the heart of *Panulirus* to adrenalin, but certain hearts showed a striking response to adrenalin 10^{-6} to 10^{-5} . Figure 4 is a record showing the effect of perfusing a heart for 2 minutes with adrenalin 10^{-5} . Marked acceleration was followed by complete diastolic stoppage during 4 minutes of washing; then a beat of greatly increased amplitude and frequency appeared. Although the frequency rapidly returned to normal, the increased amplitude was noticeable 20 minutes after perfusion with the adrenalin had ceased.

¹ Whether a slight rise in base line is the result of a tonus change or should be interpreted as partial tetanus is not clear. Since it is generally agreed that the arthropod heart is capable of being tetanized, the marked effects seen in Figures 1, 2, and 3 may properly be termed "tetanic effects."

These effects of a relatively high concentration of adrenalin are very different from the effects produced by high concentrations of acetylcholine, thereby indicating that, even though they both have excitatory actions, they act in quite different ways

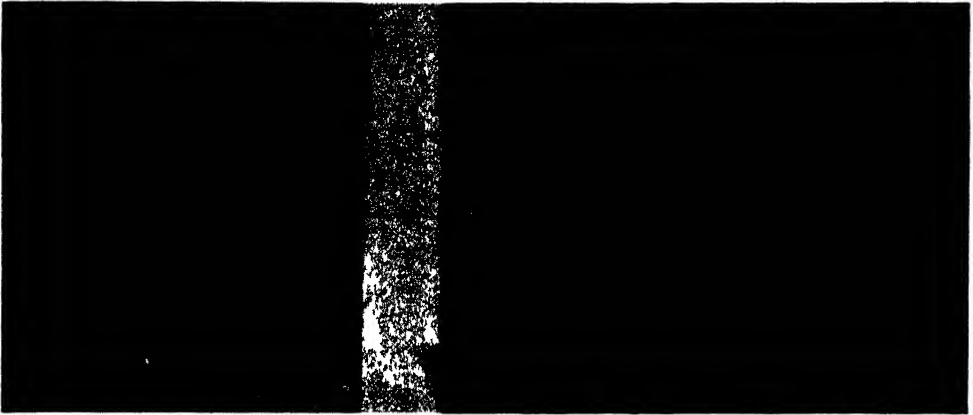


FIG. 4.—Record of the effect of adrenalin 10^{-5} . A section of the record covering a period of 4 minutes has been removed. During this period there were a few weak contractions.

THE INNERVATION OF THE HEART OF *Panulirus*

Fortunately, many features of the innervation of the decapod heart are well known, especially from the excellent work of Alexandrowicz (1932). The situation in the heart of *Panulirus argus* is almost identical with that in the closely related *Palinurus vulgaris* Latreille, which has been worked out in some detail by Alexandrowicz. By using a staining method essentially like his, it was possible to demonstrate the major features of the local nervous system of the heart. These are shown schematically in Figure 5. Unlike *Limulus* and certain other arthropods, the heart ganglion of the decapods is on the inner surface; and in order to expose it, the heart must be removed and split along the ventral side. After suitable staining, one then sees, in *Panulirus*, the view which is shown in Figure 5. Certain regions of the trunk are crossed by muscles, but the major portion is directly exposed. As in *Palinurus* and in all other marine decapods which have been studied, there are five large and four small ganglion cells. Four of the five large cells lie in a mid-dorsal region quite close to one another; the fifth is at some distance posteriorly; while the four small cells are located in the posterior portion of the trunk. In a mid-region of the trunk nine fibers may be seen, and these are grouped into two sizes—five large and four small fibers—corresponding with the two sizes of cell bodies.

Carlson (1905-6) described and figured two separate pairs of nerves arising in the thoracic ganglion of *Palinurus* and innervating the heart. He also demonstrated the effects of stimulating the heart by way of these nerves and found one pair to be inhibitory and the other acceleratory in function. Alexandrowicz could find only a single pair of nerves running to the heart ganglion of this same form, but they contained fibers of two sizes, suggesting a difference in function. The question of the actual number of extrinsic nerves to the heart ganglion remains to be determined, but the observations by Carlson on the functional activity of the extrinsic fibers have been abundantly confirmed.

Certain details regarding the intimate relations between the several ganglion cells remain to be worked out; but Alexandrowicz has shown that, in spite of the small number of nervous elements, the morphological situation in the decapod heart is very complex, and it follows that the functional situation is likewise complex. Transection of the nerve trunk in a mid-region stops the regular beat of the heart. The ganglion may then be considered the pacemaker for the heart, as in *Limulus*, which has been so extensively studied.

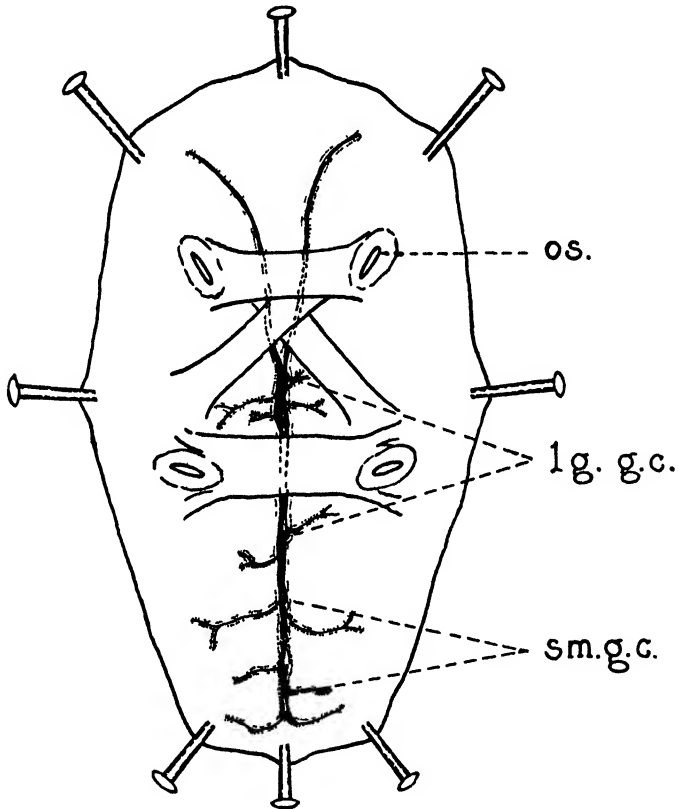


FIG 5 -View of the heart opened along the ventral side and pinned out to show the nerve trunk and ganglion cells, as seen after staining with methylene blue. Four muscles which run between the ostia (*os*) cross the heart ganglion. The five large ganglion cells (*lg g.c.*) are shown with disproportionately large cell bodies. The four small ganglion cells (*sm g.c.*) are situated toward the posterior end of the heart.

One of the interesting questions regarding pacemaker activity in the decapod heart is the role which the contracting muscle may play in regulating the discharge from pacemaker cells. Alexandrowicz (1932) presents some evidence indicating a return connection from muscles to ganglion cells by way of "dendrites" and suggests that "the impulses conveyed by the dendrites from the muscles would serve for the self-regulation of the rhythmical action of the neuromuscular apparatus of the heart." If such a situation as this does exist in the decapod heart, then any substance which (1) affected the pacemaker activity of

the ganglion cells, (2) facilitated transmission at myoneural junctions, or (3) acted on muscle cells directly might modify the frequency and amplitude of beat. If the local nervous system of the decapod heart is cholinergic, as seems to be the case, then perfused acetylcholine would probably have its greatest effect at myoneural junctions. Adrenalin, on the other hand, might act directly on the muscle cells. However, these are only suggestions, and added information will be necessary before final conclusions can be reached.

SUMMARY

1. Both acetylcholine and adrenalin have an excitatory action on the isolated heart of *Panulirus argus*, the spiny lobster.
2. The actions of eserine and atropine are such as to indicate that the local nervous system of this heart is cholinergic.
3. Low concentrations of acetylcholine cause an increase in frequency and amplitude of beat. High concentrations produce partial or complete tetanus and systolic stoppage.
4. High concentrations of adrenalin produce an increase in frequency and amplitude of beat and occasionally diastolic stoppage but not the tonus changes and tetanus produced by acetylcholine.
5. The different responses to acetylcholine and adrenalin suggest that these substances act on different mechanisms within the heart.
6. The number and arrangement of the ganglion cells and nerve fibers in the heart of *Panulirus* is similar to that in the majority of decapod crustaceans. Five large and four small cells have complex anatomical and physiological relations to one another and to the muscles.

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ON THE KILLING ACTION OF OPTICALLY ISOMERIC NICOTINES IN RELATION TO PROBLEMS OF EVOLUTION OF THE NERVOUS SYSTEM IN ANIMALS

(Ten figures)

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I. INTRODUCTION

IN THE present work the analysis of the killing action of optically isomeric nicotine on different animals was used as a method permitting a new approach to the study of evolution of their nervous systems. The investigation was carried out with vertebrates and with different groups of both fresh-water and marine invertebrates; experiments with the latter were made at the Karadag Biological Station on the Black Sea.

In the first place, certain questions of method arise. Pictet and Rotschy for the first time prepared pure dextrorotatory (unnatural) nicotine in 1904, tested its toxicity on rabbits and guinea pigs, and ascertained that it was less efficient than the natural *l*-nicotine. At the same time, they expressed the view, which subsequently was adopted by a number of compilers, that the mechanism of the toxic action of the dextro-isomer might be different from that of the laevo form, as the symptoms of poisoning with different isomers of nicotine are different. It is evident that, in order to confirm or to reject this hypothesis, exact quantitative investigations are necessary, which were not carried out by Pictet and Rotschy at the time of their first preliminary observations. The necessity of such investigations with optically isomeric nicotine was pointed out by Cushny (1926), and more recently Macht (1929) published a paper on the pharmacologic synergism of stereoisomeric nicotine. The conclusion reached by him is that "an individual cell may possess receptor groups of a laevo and dextro type, and a mixture of two stereoisomers would thus have a double point of attack in place of a single one in case only one of the optic isomers was used." This important conclusion is, however, based on very few observations, which were not treated statistically, from which the author infers that the toxic action of the mixture of *l*- and *dl*-nicotine proves to be stronger than the simple additive action of these isomers. Statistically this conclusion does not hold good because of the very small number of experiments and because of the fact that the author did not attempt to obtain a concentration-toxicity curve which would permit quantitative calculations. Moreover, the preparation of *dl*-nicotine is little suitable for such experiments, as it usually contains an admixture of hydronicotine, which influences its physiological effect (Gause, 1936). Therefore, only the purest dextro-nicotine, obtained by repeated recrystallizations with laevotartaric acid, must be employed here.

The results of our experiments do not give any indications of the existence of any basic differences in the actual mechanism of toxic action of the optic isomers of nicotine. The dextro-isomer, in accordance with the data of Pictet and Rotschy, proves to be less poisonous for vertebrates than the laevo form; but the dynamics of increase of toxicity with concentration is identical in both, and consequently they seem to act on the same link in the system of biological processes but with different speeds in connection with the

spatial factor. This conclusion is also confirmed by the fact that complete identity of temperature characteristics of toxic action of dextro- and laevo-nicotines was observed by us in cold-blooded animals (fishes and tadpoles); such an identity could not exist if the stereoisomers differed as to the mechanism of their action.

This work is based upon the following observations. Higher animals have evidently some specific spatially sensitive substance which is affected by the dextro- and laevo-isomers of nicotine with a difference in rate, the nature of which we shall try to identify exactly. Protozoa do not possess, as our observations have shown, such a spatially sensitive substance; and the dextro- and laevo-isomers of nicotine are equally toxic for them. The question arises: When in evolution did this specific, spatially sensitive substance appear first, and what is its nature? It is interesting to point out that Greenwood, as early as 1890, carried out an extensive comparative investigation of the action of common laevorotatory nicotine on invertebrates, attempting to establish parallelism in the character of the action of this alkaloid which affects, as is known, the nervous systems of animals, with the evolution of their nervous systems. On the basis of purely qualitative observations he concluded:

The toxic effect of nicotine on any organism is determined mainly by the degree of development of the nervous system. Thus for *Amoeba* it cannot be regarded as exciting or paralyzing; it is rather inimical to continued healthy life. As soon as any structural complexity is reached, the action of nicotine is discriminating, and discriminating in such a fashion that the nervous actions which are the expression of automatism, which imply co-ordination of impulses, are stopped first. This is seen dimly in *Hydra*, and it is more pronounced among the Medusae. When structural development goes farther, the selective action of nicotine is traced readily, as for example in *Palaemon*.

Greenwood writes further that "animals which have enough in common to stand near each other in classification may yet react differently upon nicotine, each according to what I may perhaps call its own balance of organisation."

The data obtained by us in a comparative investigation of the toxic action of optically isomeric nicotines differ from those of Greenwood in their greater stability. This is apparently the consequence of the great technical advantages which the work with two optical isomers presents, as compared to the investigation of the action on different animals of a single isomer of some substance. In the latter case we shall never be able to conclude definitely whether the increased sensitiveness to a given preparation in one species of animals is the consequence of the appearance in it of some specific "receptive substance" or whether it results from altered conditions of the penetration of the preparation into the animal, depending upon the form and size of its body, etc. Interpretation of the results is, therefore, possible only in terms of the balance of organization of the animal. But, as experience shows, when we employ two optical isomers identical in all their physicochemical properties, we are able to ascertain the nonspecific differences of sensitiveness of animals to a given substance. In case of such nonspecific differences the effectiveness of dextro- and laevo-isomers differs in equal degree, and the relation between their toxicities, or the "spatial coefficient" of the toxic action, does not change. In case of alterations in the receptive substance, however, we immediately detect alteration in the spatial coefficient of the toxic action.¹

¹ These considerations hold true for experiments with optically isomeric nicotines, and a more general theory of the principles of such an "asymmetric analysis" of the structure of living systems is reserved for further publications.

Experiments have shown that the methods applied by us permit us to detect either the "absence" or the "presence" of the specific receptive substance spatially sensitive to nicotine, which first appears in the nervous systems of higher worms and which does not undergo any considerable changes in chemical nature in the course of further evolution of animals. Investigation of the presence of this substance in different groups of invertebrates sheds some light upon certain problems of their phylogeny.

II. METHODS

Special attention was given to the purity of preparations used. We employed *l*-nicotine *purissimus* from the Schuchardt Company (Görlitz) with specific rotation attaining $[\alpha]_D = -169^\circ$. The dextro-nicotine was prepared according to Pictet and Rotschy (1904). At first the laevo-nicotine was racemized by heating nicotine sulphate, and then the dextro-isomer was removed from the racemic mixture and purified by repeated crystallization with laevotartaric acid obtained from Schuchardt. Specific rotation of our specimen of dextro-nicotine attains $[\alpha]_D = +140^\circ$.

Before each set of experiments a 10 per cent solution of nicotine in bidistilled water was prepared, then neutralized by 10 per cent HCl to pH = 8.5, and was usually also tested (in suitable dilution) on paramecia (*Paramecium caudatum*) in order to make sure that dextro- and laevo-isomers have quite equal toxicity. From this initial solution the various dilutions required were prepared—for fresh-water animals with bidistilled water, for marine animals with water of the Black Sea.

III. EXPERIMENTS ON VERTEBRATES

a) *Bird (Acanthis flammea)*.—We made injections of a neutralized solution of nicotine (0.1–0.3 per cent) into the breast muscle of *A. flammea* in quantities of a definite number of milligrams for a hundred grams of weight.

Figure 1 represents the relation of the killing time in seconds to the concentration of the introduced nicotine. It is to be pointed out that for calculation of the relative efficiency of optically isomeric nictines one should not compare their effectiveness with any arbitrarily chosen concentration of the poison. Thus, for example, with 3 mg. of nicotine for 100 gm. of weight, *d*- and *l*-isomers, as far as their toxicity is concerned, are very near one another, while with 2 mg. of nicotine the difference between them is very distinct. It is therefore obvious that for comparison of the physiological effect of optic isomers we have to employ characteristics of corresponding curves of toxicity which will not depend on the absolute concentration of the poison. The most convenient procedure is to take the constant n , which characterizes the minimal lethal concentration of the poison. In *A. flammea* n equals 0.8 mg. for *l*-nicotine, while for *d*-nicotine n equals 2.5 mg. The spatial coefficient of the toxic action (α), representing the ratio of minimal lethal concentration of *d*-isomer to minimal lethal concentration of *l*-isomer, is 3.12.

In order to get an idea of the dynamics of the toxic action, the curves of toxicity should be converted into straight lines, by plotting the logarithms of the killing time ($\log y$) opposite the logarithms of the corresponding effective concentrations of the poison $[\log (x - n)]$. According to Figure 2, calculated on the basis of ordinates taken from the continuous curves of Figure 1, the slopes of the straight lines, characterizing the dynamics of the increase of toxicity with concentration, are identical for both optic isomers of nicotine; consequently, we have some right to assume the identity of the mecha-

nism of the toxic action of optically isomeric nictines. In our experiments we could not discover any distinct difference in the symptoms of poisoning with the two isomers.

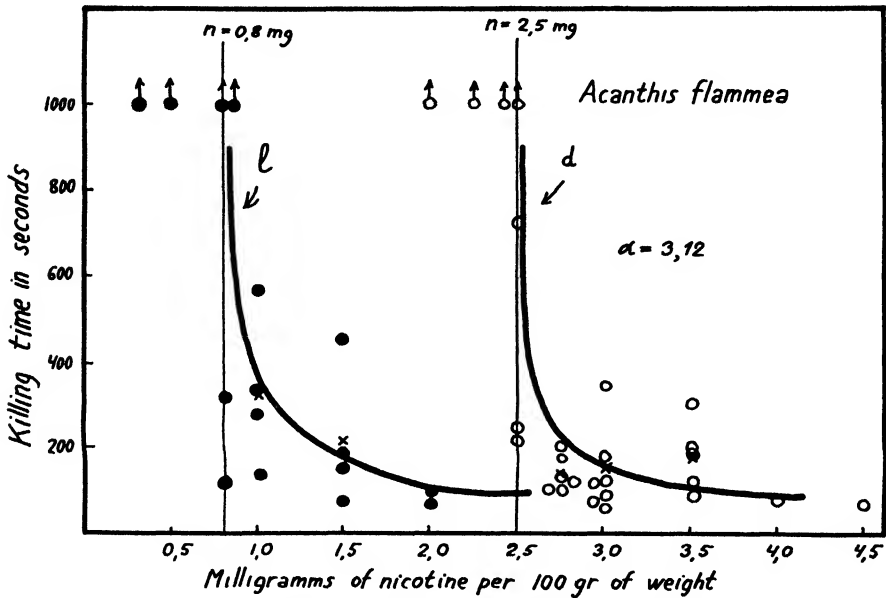


FIG. 1. — Toxic action of optically isomeric nictines on *Acanthis flammea*

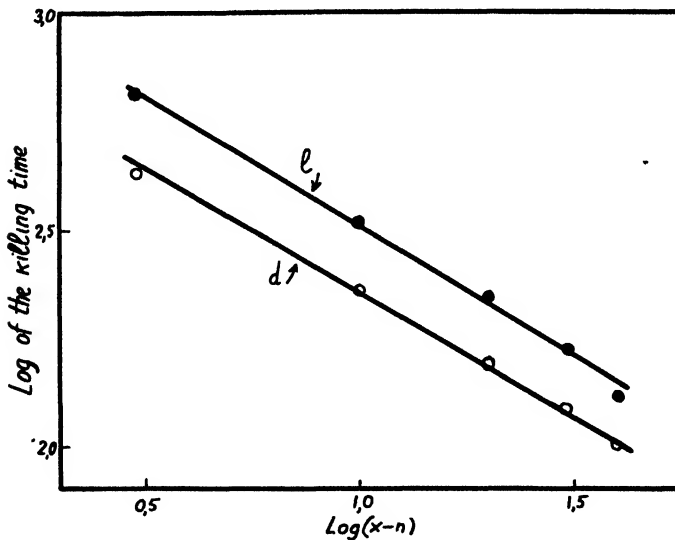


FIG. 2. — Logarithmic curves of toxicity of optically isomeric nictines for *Acanthis flammea*

b) *Lizard (Lacerta viridis)*.—The injection of the solution of nicotine was made into the muscle of the right hip. The data obtained are given in Figure 3. In consequence of

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great variability of separate observations it is necessary to make here an exact calculation of minimal lethal concentrations of the poison for both optic isomers (threshold values of n). The threshold represents a concentration of poison below which less than 50 per cent of the individuals perish and above which over 50 per cent perish. For every concentration of nicotine the percentage of individuals killed was computed. On the basis of the curve relating the percentage killed to the concentration of the poison, the abscissa (concentration of poison) was determined graphically when the ordinate showed that 50 per cent had perished. Thus, thresholds of poisoning were found to be: for the laevo-isomer, 5.6 mg., and for the dextro-isomer, 13.5 mg., per 100 gm. of weight. The coefficient a is here 2.41.

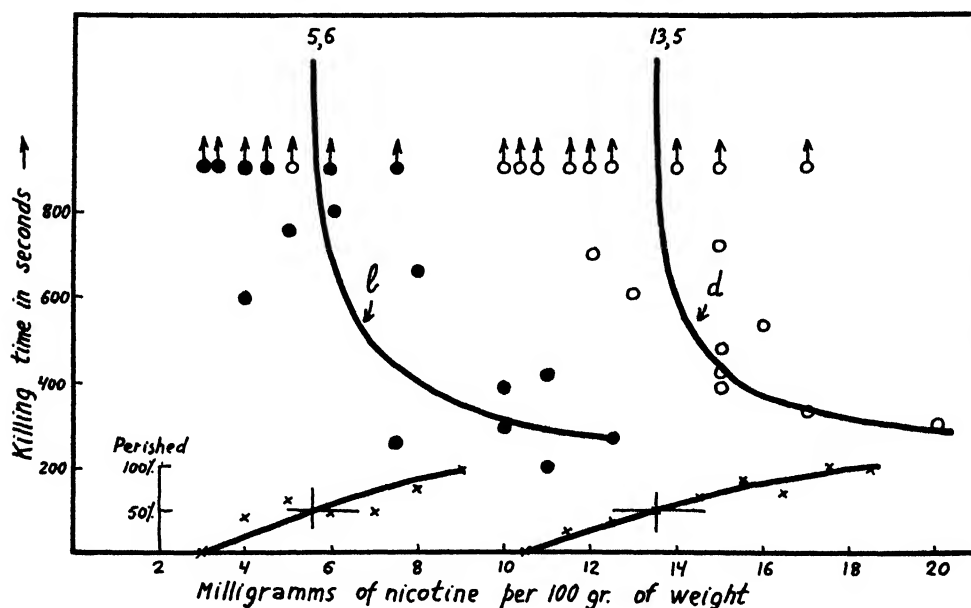


FIG. 3.—Toxic action of optically isomeric nictines on *Lacerta viridis*. Upper curves refer to killing time in seconds; lower curves refer to percentage of perished individuals

c) *Tadpoles (Rana temporaria)*.—Experiments were made with tadpoles (13 mm. long) reared in the laboratory from eggs laid in the open. Tadpoles were placed in neutralized solutions of nicotine of different concentration, prepared with bidistilled water. Each point on the curve of toxicity traced in Figure 4 represents the mean of five observations. The threshold concentrations of poison, as shown in Figure 4, are here very low; and for their exact calculation we have employed the following method. Treating independently the curves of toxicity for the dextro- and laevo-isomers, we at first determined the approximate values of n on the basis of experimental data. Then, working with logarithmic curves, n was selected more exactly, so as to obtain a strictly linear relation between $\log y$ and $\log (x - n)$. In this procedure we evidently implied that the divergence from the linear type of relation, which we are correcting, is due to the incorrectly selected parameter n , but that the nature of the relation of the toxicity to concentration is actually described by the empiric curve of the type $y = k/(x - n)^m$. This as-

sumption follows from the whole experience of modern quantitative toxicology, and with the appropriate selection of parameter n we have actually always obtained a linear relation between $\log y$ and $\log (x - n)$.

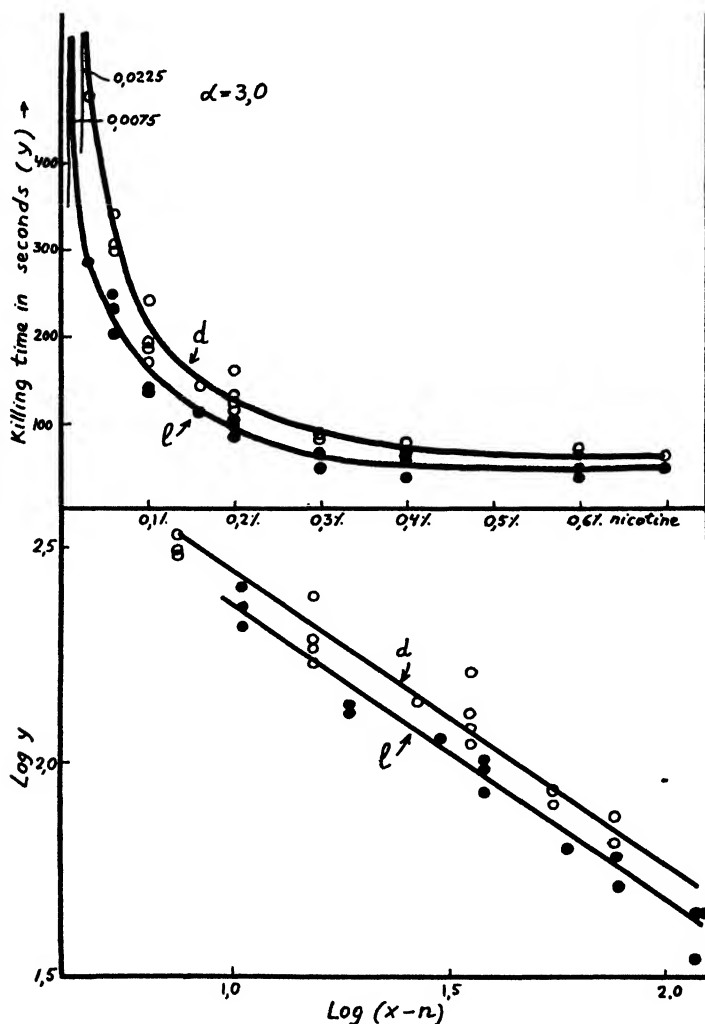


FIG. 4.—Toxic action of optically isomeric nicotines on tadpoles (*Rana temporaria*). Lower curves are logarithmic.

Figure 4 shows that the threshold concentration of the *l*-isomer is 0.0075 per cent and that of the *d*-isomer is 0.0225 per cent. Here the coefficient α equals 3.0. It is very important to point out that the relative position of the curves of toxicity of optic isomers in tadpoles differs sharply at first sight from that observed in lizards. Owing to very small absolute values of threshold concentrations, the curves of toxicity of optic isomers in

tadpoles seem very much approximated, as compared to the relative position of these curves in the lizard. However, the relation between thresholds which finds its expression in the spatial coefficient of the toxic action of nicotine (α) proves to be practically equal in all animals heretofore examined. In conclusion it may be added that in tadpoles the slopes of the logarithmic curves of toxicity are identical in both optically isomeric nico-
tines.

We may now pass to temperature coefficients of toxic action of optic isomers of nicotine upon tadpoles. The experiments consisted in placing 0.1 per cent solution of nico-

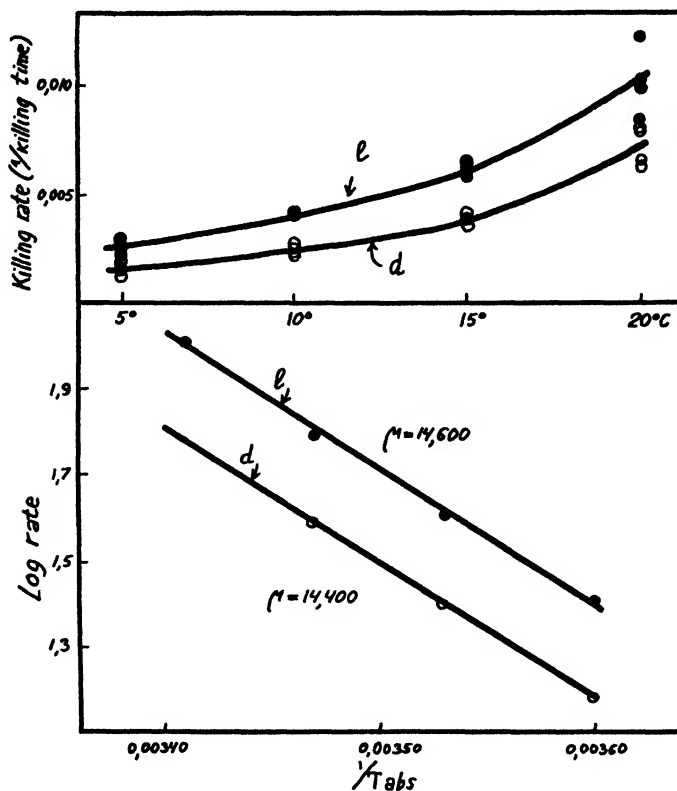


FIG. 5.—The effect of temperature on toxic action of optically isomeric nico-
tines upon tadpoles (*Rana temporaria*).

tine in experimental vessels in a water bath with controlled temperature. After temperature equilibrium was attained, tadpoles were placed in the solution of nicotine, and the killing time at different constant temperatures was observed. Figure 5 represents the results of 240 experiments of this kind. The upper curves show the relation of killing-rates (values inverse to corresponding killing times in seconds) to temperature. For the purpose of computation of temperature characteristics of toxic action we plotted on the lower curves of Figure 5 logarithms of the killing-rates against the inverse values of absolute temperatures. When computing logarithms of killing-rates, we made use of the mean values of speeds for each temperature. It may be stated definitely that temperature charac-

teristics of toxic action practically coincide for the laevo- ($\mu = 14,600$) and dextro- ($\mu = 14,400$) isomers of nicotine. Thus, the relation of toxic action to temperature strongly supports the view that the mechanism of toxic action is identical in optically isomeric nicotines.

d) Fish (*Leuciscus idus* var. *orfus*).—Experiments with brood of *L. idus* were made according to the same plan as with tadpoles. Each point on Figure 6 represents the mean

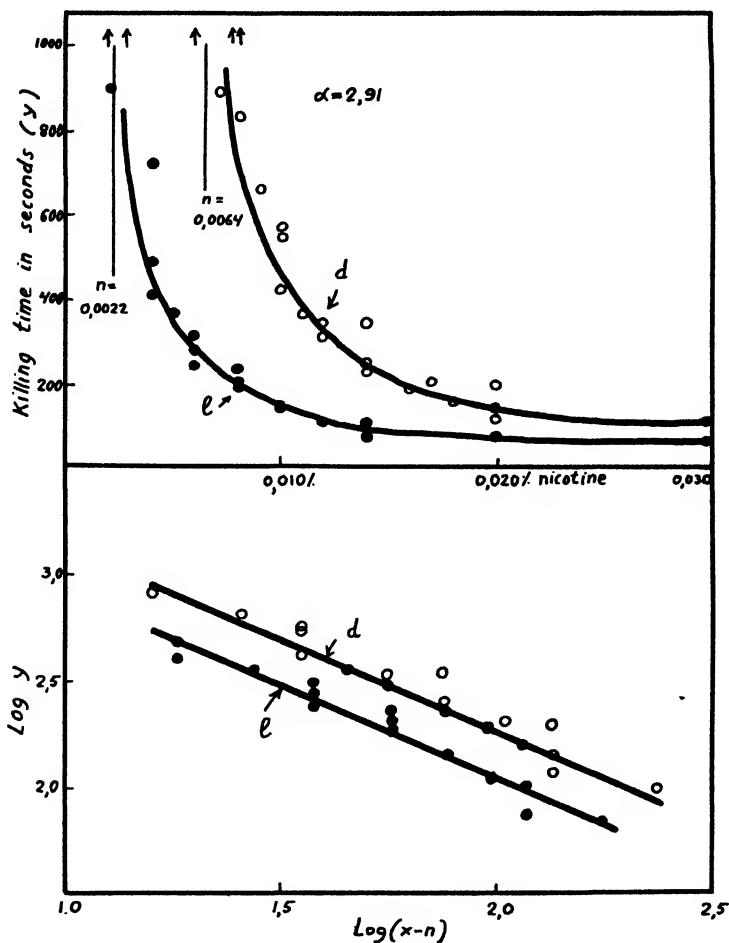


FIG. 6.—Toxic action of optically isomeric nicotines on *Leuciscus idus*. Lower curves are logarithmic

of five observations. It may be pointed out that the spatial coefficient of toxic action of nicotine (α) here equals 2.91. Slopes of logarithmic curves of toxicity coincide in optical antipodes.

Figure 7 represents the results of experiments on temperature characteristics of the toxic action of isomers of nicotine. For these experiments practically isotoxic concentrations of antipodes were selected, i.e., *l*-nicotine in the concentration 0.007 per cent and *d*-nicotine in the concentration 0.014 per cent. Altogether, 120 such experiments were

made. Their results, according to Figure 7, demonstrate the identity of temperature characteristics of intoxication of *L. idus* with dextro- and laevo-isomers of nicotine.²

e) *Fish (Lebistes reticulatus)*.—The last representative of vertebrates studied by us was the brood of *L. reticulatus*. Each point on Figure 8 is the mean of three observations. Coefficient α here equals 2.4.

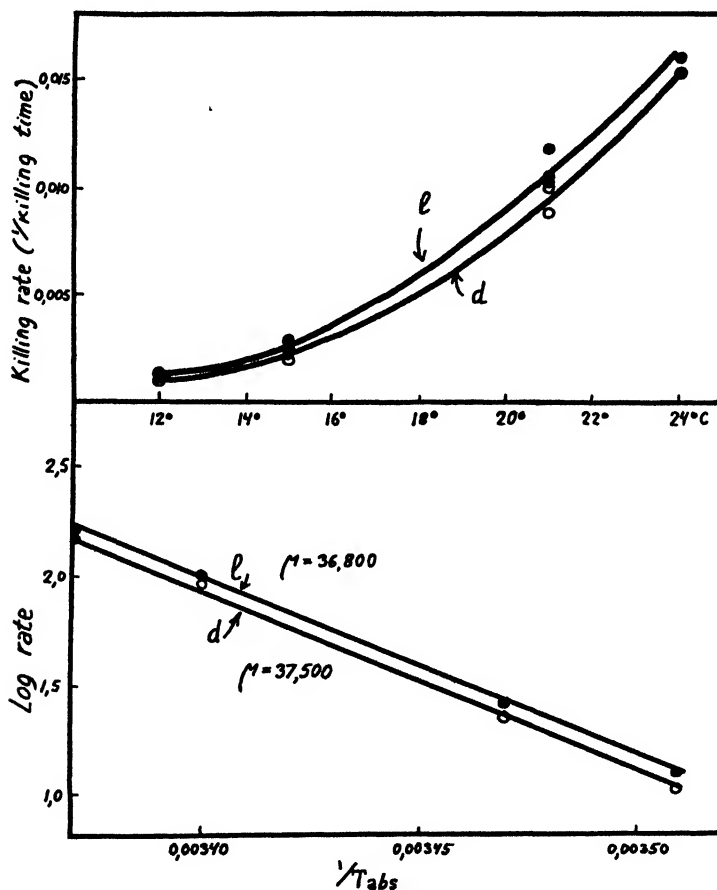


FIG. 7.—The effect of temperature on toxic action of optically isomeric nictines upon *Leuciscus idus*

f) *Conclusions*.—Experiments on vertebrates permit us to make the following two essential conclusions. First, the mechanism of toxic action of dextro- and laevo-isomers of nicotine prove to be equal. This is demonstrated by the identity of dynamics of toxic action (identity in slopes of logarithmic curves of toxicity) and the identity of temperature characteristics of toxic action in optically isomeric nictines. Second, spatial co-

² We cannot, as yet, give any explanation of the fact that the temperature characteristics of intoxication in tadpoles are of the order of 14,000 and that in the *L. idus* of the order of 37,000.

efficients of the toxic action of nicotine prove to be of one and the same order of magnitude in all investigated animals:

<i>Acanthis flammea</i>	$\alpha = 3.1$
<i>Lacerta viridis</i>	$\alpha = 2.4$
<i>Rana temporaria</i>	$\alpha = 3.0$
<i>Leuciscus idus</i>	$\alpha = 2.9$
<i>Lebistes reticulatus</i>	$\alpha = 2.4$
Mean	$\alpha = 2.8$

The mean value of this coefficient is 2.8. The first and the second conclusions, being independent, agree well with one another. Inasmuch as the mechanism of toxic action is identical in optically isomeric nictines, we can judge, by the difference of their effects,

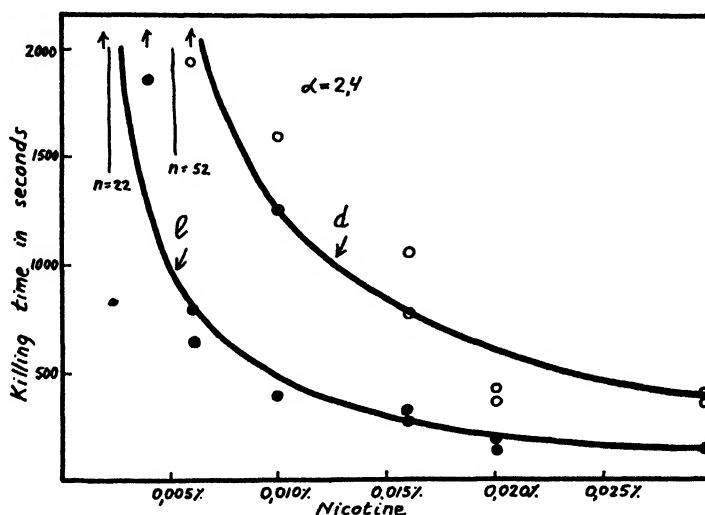


FIG. 8.—Toxic action of optically isomeric nictines on *Lebistes reticulatus*

the spatial properties of some specific receptive substance; as the difference of effects remains constant, one can conclude that the chemical nature of the receptive substance within the vertebrates remains also essentially constant.

This last result is of some interest in connection with the method of investigation used. It represents an experimental proof of the fact that the conditions of the penetration of nicotine into the animal do not affect significantly the value of the spatial coefficient. Nicotine was introduced into the muscle of *Lacerta* while *Lebistes* were immersed in the solution of nicotine; nevertheless, the spatial coefficients of toxic action of nicotine are identical in these two animals. Absolute sensitiveness to nicotine in *Acanthis* is considerably higher than that in *Lacerta* (0.8 mg. per 100 gm. of weight, as compared to 5.6 mg. per 100 gm. of weight), but practically it does not influence the spatial coefficient of the action of nicotine. It is therefore evident that the spatial coefficient (α) gives us an opportunity to judge of the chemical nature of the specific receptive substance.

IV. EXPERIMENTS ON INVERTEBRATES

The results of experiments with fresh-water and marine invertebrates are given in Table 1. For each species we recorded specific symptoms of intoxication (taking into consideration the moment of the cessation of voluntary movements) and plotted curves

TABLE 1
TOXIC ACTION OF OPTICALLY ISOMERIC NICOTINES UPON INVERTEBRATES

Animal	Fresh Water or Marine	Range of Concen- trations Studied (Percentage)	No. of Ex- periments	Results	
Protozoa:					
1. <i>Paramecium caudatum</i>	f.w.	0.6 - 1.2	80		
2. <i>Euplotes patella</i>	f.w.	1.0 - 2.6	34		
3. <i>Stentor coeruleus</i>	f.w.	0.4 - 0.8	24		
4. <i>Spirostomum ambiguum</i>	f.w.	0.2 - 0.6	24		
Coelenterata:					
5. <i>Hydra fusca</i>	f.w.	1.0 - 2.0	24		
6. <i>Cladonema radiatum</i>	mar.	0.2 - 0.5	12		
Platyhelminthes, Turbellaria:					
7. <i>Polycelis nigra</i>	f.w.	0.3 - 0.8	34		d- and l-nicotines are isotoxic
8. <i>Phaenocora</i> sp.	f.w.	0.2 - 1.0	32		
9. <i>Dalyella brevimana</i>	f.w.	0.4 - 1.0	30		
10. <i>Procerodes lobata</i>	mar.	0.3 - 0.8	10		
11. <i>Leptoplana tremellaris</i>	mar.	0.01 - 0.05	16		
Rotatoria:					
12. <i>Euchlanis triquetra</i>	f.w.	0.02 - 0.1	20		
13. <i>Rotifer vulgaris</i>	f.w.	0.06 - 0.2	24		
Nemertini:					
14. <i>Lineus lacteus</i>	mar.	0.4 - 1.0	12		
Annelida:					
15. <i>Saccocirrus papillocercus</i>	mar.	0.1 - 0.7	19	l-nicotine is more toxic	
16. <i>Perinereis cultrifera</i>	mar.	0.3 - 1.0	18		
17. <i>Arenicola grubii</i>	mar.	0.75	8		
18. <i>Pristina longiseta</i>	f.w.	0.1 - 1.0	38		
19. <i>Limnodrilus hoffmeisteri</i>	f.w.	0.025 - 1.6	136		
20. <i>Helobdella stagnalis</i>	f.w.	0.0002 - 0.007	78		
21. <i>Nais communis</i>	f.w.	0.1 - 0.6	60		
22. <i>Chaetogaster langi</i>	f.w.	0.02 - 0.2	54		
23. <i>Stylaria lacustris</i>	f.w.	0.3 - 0.6	20		
24. <i>Aelosoma variegatum</i>	f.w.	0.2 - 0.6	62		
25. <i>A. hemprichi</i>	f.w.	0.2 - 0.6	58		
Chaetognatha:					
26. <i>Sagitta setosa</i>	mar.	0.025 - 0.10	16		a = 2.7
Arthropoda:					
27. <i>Daphnia magna</i>	f.w.	1.2 - 10.0	124	d- and l-nicotines are isotoxic	
28. <i>Cyclops serrulatus</i>	f.w.	0.25 - 1.0	54		
29. <i>Gammarus marinus</i>	mar.	0.8 - 1.0	10		
30. <i>Drosophila melanogaster</i> (two- day-old larvae were immersed in nicotine solutions)		0.4 - 1.0	30		

relating killing time in seconds to the corresponding concentration of nicotine. A part of these curves is represented on Figures 9 and 10.

Table 1 shows that all the invertebrates examined by us can be divided into two distinct groups. For those belonging to the first group the dextro- and laevo-isomers of nicotine are equally toxic, and the curves of toxicity of optical antipodes fully coincide with one another (Fig. 9). For the members of the second group the laevo-isomer of nicotine is more toxic than the dextro-isomer (Fig. 10). The one or the other attitude toward opti-

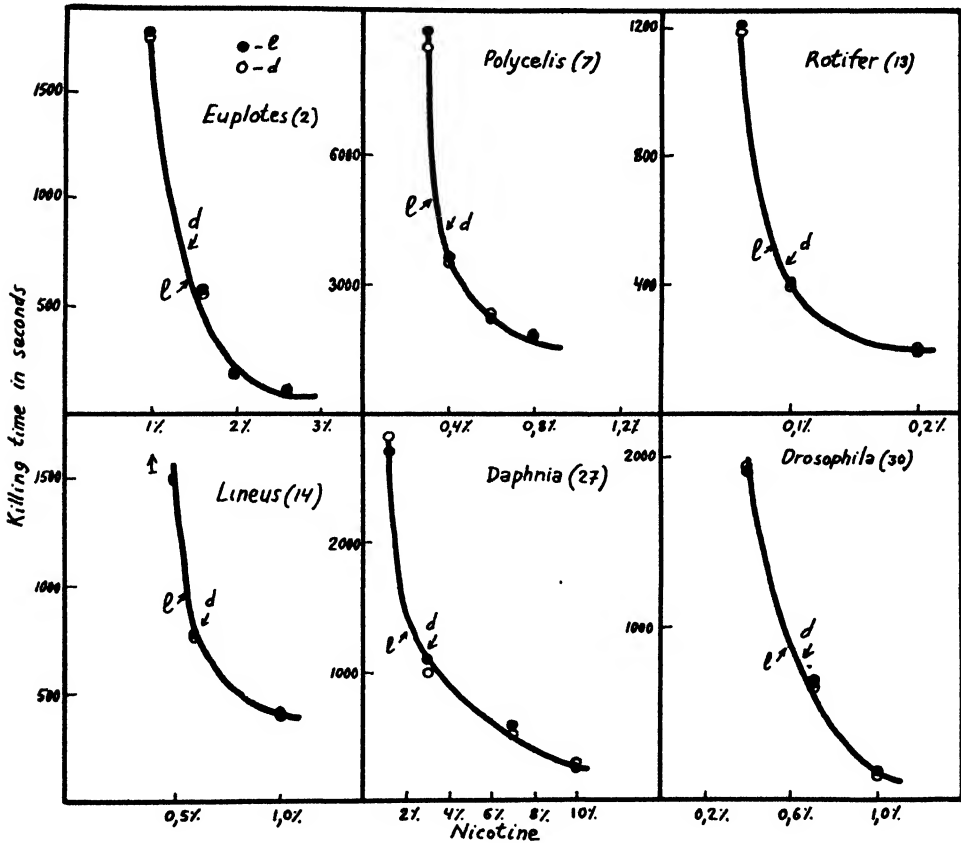


FIG. 9.—Toxic action of optically isomeric nicotines upon some invertebrates

cally isomeric nicotines appears to be specific for the large systematic groups of animals, and within these groups does not disclose any variations.

Examining Table 1 more closely, we see that all the representatives of Protozoa, Coelenterata, Turbellaria, Rotatoria, and Nemertini studied by us belong to the first group of animals. It means that they are devoid of a spatially specific receptive substance to intoxication by nicotine. It is noteworthy that in invertebrates the spatial coefficient of the action of antipodes is not affected by the differences in the absolute sensitiveness to nicotine peculiar to separate species, as has been already recorded for verte-

brates. Thus, for example, *Leptoplana* is considerably more sensitive to nicotine than *Procerodes*, but both these turbellarians are characterized by equal effect of dextro- and laevo-isomers of nicotine. The number of such examples could be considerably augmented.

A stronger effect of the laevo-isomer of nicotine is met with for the first time in Annelida, and remarkably in Archiannelida (*Saccocirrus*), Polychaeta, and Oligochaeta. Then it is observed in primitive representatives of Deuterostomia (*Sagitta setosa*). In Arthropoda

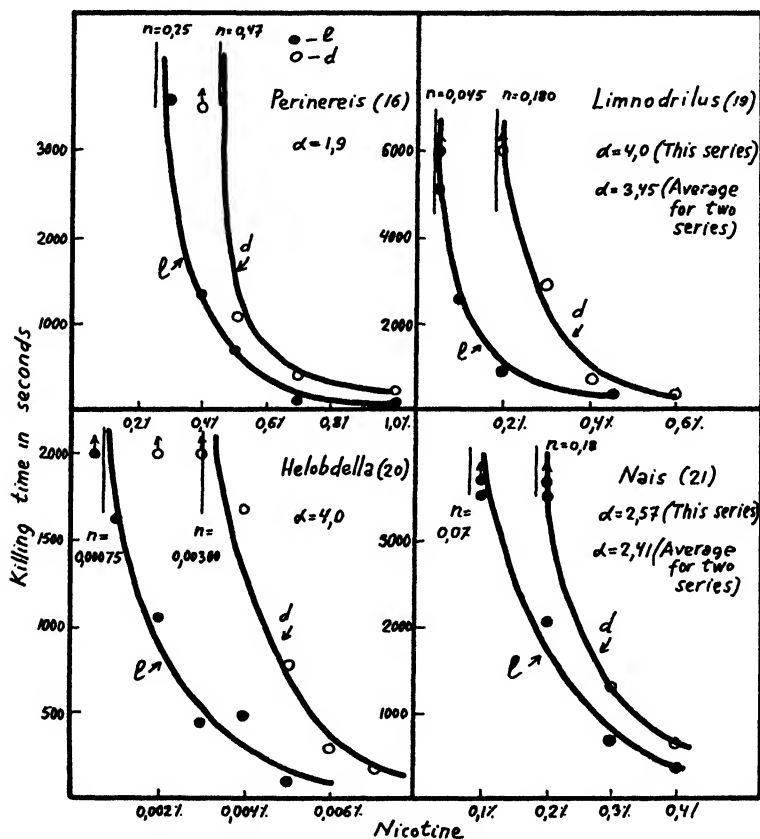


FIG. 10. -Toxic action of optically isomeric nictines upon some invertebrates

(Crustacea and Insecta) this effect is absent, and here again we find equal toxicity of dextro- and laevo-isomers of nicotine.

Let us now turn to the following question: Is the excess of the efficiency of laevo-isomer over that of the dextro-isomer, in those invertebrates possessing the spatial effect, of the same order of magnitude as in the vertebrates? For invertebrates we have:

<i>Saccocirrus papillocercus</i>	$\alpha=2.3$
<i>Perinereis cultrifera</i>	$\alpha=1.9$
<i>Pristina longiseta</i>	$\alpha=2.1$

<i>Limnodrilus hoffmeisteri</i>	$\alpha = 3.4$
<i>Helobdella stagnalis</i>	$\alpha = 4.0$
<i>Nais communis</i>	$\alpha = 2.4$
<i>Chaetogaster langi</i>	$\alpha = 3.1$
<i>Aelosoma variegatum</i>	$\alpha = 1.8$
<i>A. hemprichi</i>	$\alpha = 1.8$
<i>Squilla setosa</i>	$\alpha = 2.7$
Mean.....	$\alpha = 2.6$

When considering these figures, one must have in view that inaccuracies in determining the value of the coefficient α may be considerable. Thus, in case of two independent determinations we found: in *Nais*, 2.57 and 2.25; in *Limnodrilus*, 2.9 and 4.0; in *Aelosoma variegatum*, 1.79 and 1.74; and in *Aelosoma hemprichi*, 1.50 and 2.18. In the light of these observations we may infer that the average coefficient α in invertebrates (2.6) and in vertebrates (2.8) is a magnitude of the same order.

V. DISCUSSION

The data obtained by us show that in Protozoa, Coelenterata, Turbellaria, Rotatoria, and Nemertini the spatially specific receptive substance affected by nicotine is absent; both optical isomers are here equally toxic. Annelida, Chaetognatha, and vertebrates possess the spatial receptor, while in Arthropoda it is again absent.

In order to try to comprehend the nature of the receptive substance we have to consider several groups of facts. First of all, identity of the spatial coefficient and, consequently, identity of the receptive substance is observed in invertebrates and in vertebrates in spite of essential differences in morphology of their nervous systems, which, as it is known, is affected by nicotine. Consequently, we have to interpret the receptive substance as some widely spread chemical constituent. However, this chemical constituent is not an obligatory component of every nervous system. This receptive substance is absent from the nervous systems of lower invertebrates (Turbellaria and Nemertini), although they show considerable differentiation.

The next step toward understanding the nature of the receptive substance may be made by examining current views concerning the mechanism of nicotine intoxication. In a number of papers Thomas and Franke (1924, 1928, 1933) have shown that paralysis of peripheral neuromuscular junctions of respiratory muscles is the cause of death of higher animals in acute nicotine poisoning. This view was confirmed by Gold and Brown (1935). The action of nicotine upon neuromuscular junctions brings us directly to the classical observations of Langley (1905), who found that in the system "neuro-muscular junction" there is certain sensitive "receptive substance" which is the first to be affected by nicotine.

Let us try to formulate a hypothesis of the possible nature of the "receptive substance." Since the classical works of Loewi, it is known that there is a chemical step in the transmission of impulses from nerves to effectors according to the scheme: (1) nerve impulse \rightarrow (2) chemical mediator \rightarrow (3) receptive substance \rightarrow (4) specific response (for literature see Cannon and Rosenbluth, 1937). There are some indications that the chemical mediator of voluntary muscles of higher animals is acetylcholine; and its action on the receptive substance of the effector in this case reminds one of the action of nico-

tine: in small doses it excites, and in large doses paralyzes.³ According to current views, nicotine, in acute poisoning of the animal, affects in some irreversible way the receptive substance, so that acetylcholine mediation cannot act any more.

All these suggestions can be expressed more definitely in the following way. Nicotine (at least in experiments of our type) acts upon neuro-effector synapses of voluntary muscles. In its action it reminds one of acetylcholine, a substance transmitting excitement in these synapses. Consequently, the receptive substance in nicotine poisoning must have some close relation to the receptive substance for chemical mediation. Our experiments permit us to divide animals into two groups, differing as regards the nature of the receptive substance affected by nicotine. May it not prove that animals possessing receptive substance structurally active and structurally inactive to nicotine differ from one another in the receptive substance of chemical mediation and, consequently, in the mechanism of transmission of nervous impulses?

In order to answer this question, we have to examine the data of physiologists concerning the distribution of acetylcholine in different groups of invertebrates. In the first place we must point out that the method of identification of acetylcholine is subject to criticism. The presence of acetylcholine is ascertained by the action of the extract under examination on different organs while, as Cannon and Rosenbluth (1937) pointed out, none of these organs is strictly specific and in this way the distinguishing between their reaction to acetylcholine and to other substances become difficult. The results may be regarded as reliable if, with several different methods, quantitatively coinciding data are obtained. This condition, however, is seldom fulfilled. Therefore, communications concerning this problem appearing in the literature must be critically viewed. The most extensive and elaborate investigations were carried out by Bacq (1935) at the Zoological Station of Naples. First, he did not discover in the tissues of different coelenterates either acetylcholine or the enzyme destroying it, choline-esterase. The muscles of annelids and of lower Deuterostomia (*Holothuria*) contain acetylcholine and choline-esterase. Highly interesting but yet somewhat contradictory are the data concerning Crustacea and Insecta. In the muscles of Crustacea was found only a small quantity of acetylcholine. Hence, Bacq inferred that the transmission of impulses from the motor nerve to the muscle in Crustacea is not accomplished by means of acetylcholine mediation. He insisted upon this at the conference devoted to this problem in Cambridge, England, in June, 1937. On the other hand, there are some preliminary communications concerning the presence of choline-esterase in ganglia of Crustacea (Nachmansohn, 1937). However, neuro-effector synapses of the muscles of Crustacea are not typical acetylcholine systems, if only for the reason that they are extremely insensitive to external acetylcholine.

In comparing Bacq's observations with our results (see Table 2), it is quite apparent that the optically active receptor for nicotine poisoning is found where classical acetylcholine mediation is observed and is absent where the classical acetylcholine mediation is not detected. In six cases we have to do with a complete coincidence of these two series of independently obtained results. If our suggestions are correct, the spatial effect of

³ A parallelism in the action of nicotine and acetylcholine can be observed on our experimental material. It is known that the leech is extremely sensitive to acetylcholine and reacts to insignificant traces of it (Minz, 1932). According to Table 1, the leech, *Helobdella stagnalis*, stands out sharply from all other animals by its extreme sensitiveness to nicotine, which proves to be toxic in concentration 0.00075 per cent.

nicotine could be employed to detect the presence of the specific receptor characteristic for the acetylcholine system in the neuro-effector synapse of voluntary muscles.

In conclusion we must examine the data obtained from the point of view of some problems of the phylogeny of invertebrates. If one agrees to consider the presence of spatial effect in nicotine poisoning and the presence of the typical acetylcholine system in neuro-effector synapses of voluntary muscles as equivalents, the following general picture may be sketched. In Protozoa, Coelenterata, Turbellaria, and related groups (Rotatoria and Nemertini) the acetylcholine system has not yet appeared. The similarity in this respect of Nemertini to Turbellaria may be considered as another proof in favor of the origin of Nemertini from turbellarian ancestors. The acetylcholine system appears for the first time in annelids (it is already present in archiannelids) and remains through lower Deuterostomia (Chaetognatha and *Holothuria*) up to vertebrates. Arthropoda, evidently, have secondarily modified their acetylcholine system.

TABLE 2
DISTRIBUTION OF THE ACETYLCHOLINE MEDIATION AND
OF THE SPATIAL EFFECT IN POISONING WITH
OPTICALLY ISOMERIC NICOTINES

Groups of Animals	Spatial Effect in Poisoning with Nicotine (Gause and Smaragdova)	Acetylcholine Mediation (Bacq, 1935)
1. Coelenterata.....	Absent	Absent
2. Annelida.....	Present	Present
3. Lower Deuterostomia (<i>Holothuria</i> for acetylcholine and Chaetognatha for nicotine)...	Present	Present
4. Crustacea.....	Absent	Absent
5. Insecta.....	Absent	Absent
6. Vertebrates.....	Present	Present

To what degree may we make use of the presence of the acetylcholine system in solution of the phylogenetic problem? To this end we shall at first turn to such a substance as haemoglobin, which has several times independently arisen in evolution and evidently cannot serve to establish any phylogenetic relationships. Baldwin (1937) writes:

Haemoglobin appears to be distributed in a very haphazard fashion without the slightest regard to zoological classification. It is present in all the vertebrates, in a few holothurians, several crustaceans and at least two insects, in several lamellibranchs and one gastropod (*Planorbis*), in many annelid worms and even in two species of Platyhelminthes. For a long time it was difficult to understand how so many animals of such different kinds can produce haemoglobin, while the ability to produce the other pigments seems to be very sharply confined to particular groups of closely related animals. But it is now known that practically all living cells contain cytochrome, which like haemoglobin itself is a haem pigment, and that the haem of cytochrome is certainly very like that of haemoglobin and possibly even identical with it. Since cells in general are capable of elaborating the haem of cytochrome, it is very probable that they can also produce the haem of haemoglobin if the need arises. In the vertebrates, haemoglobin acts as a respiratory pigment in the ordinary sense, but in the invertebrates it seems to have arisen independently in many different species as an adaptation to longer or shorter periods of oxygen deficiency.

In other words, we may say that for transformation of cytochrome into related haemoglobin a single, or at least a few simple, mutations were sufficient, and these were evidently accomplished and fixed by natural selection in several independent cases.

Returning to the acetylcholine system, one can say that its distribution in animals differs basically from that of haemoglobin. The presence or absence of the acetylcholine system is a characteristic not of separate species but of large systematic groups. It is absent in all turbellarians and is present in all annelids; we have never found an exception to this rule. Through the lower Deuterostomia the acetylcholine system of worms is directly related to the acetylcholine system of vertebrates; thus, we have some right to suggest that acetylcholine system has appeared in evolution only once and has never independently arisen since that time.

The problem of the position in zoological classification of lower Deuterostomia which we consider to be a connecting link between worms and vertebrates is still open to question. Dogiel (1934) summarizes in the following manner the arguments of comparative morphology.

According to one version, Deuterostomia are so isolated from other invertebrates that they have to be derived from Coelenterata independently of worms through some as yet insufficiently known stages. The weak point of this hypothesis is that it demands to accept an excessively sharp transition from coelenterates immediately to the representatives of higher animals possessing coelom, blood-vessel system, etc. According to other views, the roots of Deuterostomia have to be found among higher worms, namely, in the group of Vermidea. Such a conclusion is supported by a number of anatomical facts. However, the difference between Deuterostomia and Vermidea is so great that the origin of the former from the latter has to be considered not quite proved.

The data on distribution of acetylcholine system discussed in this paper suggest in favor of the second hypothesis.

VI. SUMMARY

1. Protozoa, Coelenterata, Turbellaria, Rotatoria, Nemertini, and Arthropoda do not possess any spatially specific receptive substance to nicotine; and both optically isomeric nicotines are, for them, equally toxic. For Annelida, Chaetognatha, and Vertebrata the laevo-isomer of nicotine is more efficient than the dextro form, and therefore these animals possess spatially specific receptive substance.

2. In considering different groups of animals, one can record a complete coincidence between the presence and the absence of the spatial effect of nicotine and the presence or absence of the typical acetylcholine system of transmission of nervous impulses. Thus, the spatial effect of nicotine could be employed to detect the presence of the acetylcholine system in neuro-effector synapses of voluntary muscles. These results are discussed in relation to some problems of the phylogeny of invertebrates.

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POSTNATAL DEVELOPMENT OF REPRODUCTIVE SYSTEM IN MALE GUINEA PIGS AND ITS RELATION TO TESTIS HORMONE SECRETION¹

(One figure)

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A STUDY of the effects of castration on the guinea pig introduced so many questions that had a bearing upon the differentiation of the reproductive system from the postnatal to the mature functional state that it was deemed advisable to investigate the prepuberal development of this system. Such a study in the rat has been carried on by Price (1936), and for the rat testis by Moore (1936). Some of the data obtained from such a study on the guinea pig are reported here, together with some of the effects of prepuberal castration, which should aid in interpreting normal reproductive development. The effects of castration in mature males will be reported elsewhere.

This paper will concern itself with the general growth and histology of the testis and accessory organs of reproduction and with the functional differentiation of the testes, prostate gland, and seminal vesicles; Cowper's gland in the guinea pig has already been studied by Heller (1932). The main points to be stressed are the time at which germ-cell differentiation occurs in the testis and the discharge of spermatozoa in the ejaculate, and the correlation of these events with evident signs of testis hormone secretion, the indications for which should be registered by changes in the accessory organs of reproduction. Effects of castration in the prepuberal males has been of some aid in the principal interpretations of some of these events.

MATERIAL AND METHODS

One hundred and eighty-five animals were utilized in this investigation: they were secured from active breeding colonies at the University of Chicago; for many of the younger animals the writer is indebted to Dr. Sewall Wright. Tissues for fixation were removed from animals immediately after death. The removal of the urethra and annexed parts permitted an adequate separation of the anterior lobe of the prostate from the middle and posterior lobes, which were not separated from each other for purposes of weight records. A torsion balance and chainomatic balance were employed for fresh gland weights. Eight different fluids for fixation and several stains were utilized in preliminary searches for specific cell organs or inclusions that might prove adequate as diagnostic indicators of the action of testis hormone on the accessory organs. Studies of Golgi material were made from preparations by the Ludford modification of the Mann-Kopsch technique (1925, 1926), both the short and long methods being employed. Paraffin sections were utilized throughout. Other details of technique will be indicated in appropriate places.

¹ This investigation has been aided by a grant from the Rockefeller Foundation to the University of Chicago. The author desires to express his appreciation to Dr. Carl R. Moore for his generous help and guidance during the course of the investigation.

GENERAL GROWTH OF REPRODUCTIVE PARTS

The growth tendencies of various parts of the male reproductive tract were determined from tissues collected from 73 animals of known ages ranging from 9 days to

TABLE 1
TABLE OF BODY AND GLAND WEIGHTS

AGE (DAYS)	NO. OF CASES	BODY WEIGHT (GRAMS)	WEIGHT (MILLIGRAMS)				
			Testes	Seminal Vesicles	Vas deferens Epididymis	Prostate Lobes	
						Middle and Posterior	Anterior
9.....	4	115	100.2	43.4	95.0	35.6	7.0
10.....	2	149	134.2	105.3	128.4	57.5	23.0
11.....	1	118	105.4	39.6	85.6	33.0	11.4
12.....	2	120	79.6	46.0	79.9	34.0	7.7
18.....	5	128	118.8	44.6	99.3	40.6	10.1
20.....	1	214	195.4	208.2	159.4	103.0	28.6
21.....	1	230	274.6	268.4	146.0	112.4	50.0
22.....	2	146	135.0	60.0	110.2	46.1	13.2
25.....	1	190	117.4	76.4	124.0	50.0	14.2
27.....	1	196	197.4	89.6	155.8	74.4	34.0
29.....	2	208	250.3	226.0	162.5	91.3	35.8
30.....	2	192	232.1	94.9	96.1	38.7	18.0
31.....	3	195	236.0	108.1	119.9	46.0	15.3
35.....	2	266	366.4	425.1	192.4	106.6	59.0
38.....	1	256	313.4	314.6	198.6	125.8	62.0
42.....	1	232	205.8	191.6	155.6	103.4	33.4
45.....	5	245	430.8	226.6	175.2	86.7	40.5
47.....	2	350	624.0	598.1	241.2	167.9	77.8
49.....	3	280	509.4	853.3	218.0	184.7	107.6
50.....	2	300	698.0	984.8	240.1	183.7	111.6
55.....	1	276	478.4	568.4	190.0	116.4	65.8
56.....	1	321	758.6	691.8	254.8	197.4	104.6
58.....	2	279	626.4	602.6	301.5	189.3	72.6
59.....	1	284	544.0	890.0	273.4	214.2	120.0
61.....	1	292	634.0	769.8	237.2	195.0	67.0
71*.....	1	270	242.2	82.8	178.2	63.4	23.8
72.....	1	308	1128.2	378.4	314.6	166.2	112.0
74.....	2	362	1389.5	510.0	324.0	222.7	101.2
77.....	2	360	1418.0	596.1	332.3	178.0	105.9
79.....	1	240	452.0	143.4	187.2	92.2	31.0
82.....	1	348	1072.0	430.0	256.6	171.4	78.0
83.....	3	377	1304.8	557.1	321.2	150.3	139.3
84.....	4	366	1161.5	673.4	303.2	181.8	102.6
85.....	2	311	1007.9	415.6	272.4	160.3	87.2
87.....	1	382	1426.8	437.4	318.2	184.6	87.4
91.....	1	305	975.7	600.0	243.2	163.6	81.5
98.....	1	299	1231.1	718.9	305.6	116.5	57.0
99.....	1	310	905.4	192.3	249.7	93.2	38.6
102.....	1	294	665.4	267.6	206.3	88.6	51.5
123.....	1	494	2239.0	1132.8	618.8	276.2	168.4
130.....	1	464	2025.8	822.2	497.2	241.4	157.0

* All animals including and above this age were ejaculated previous to killing by at least 2 days.

130 days old; by the latter age the male has attained its reproductive function. These data are presented in Table 1 and Figure 1. Seminal vesicles from animals older than
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70 days are omitted from the graph, for the reason that all animals of this age and older were subjected to an electrical stimulation to induce an ejaculation; hence, a discrepancy in growth-rate is introduced.

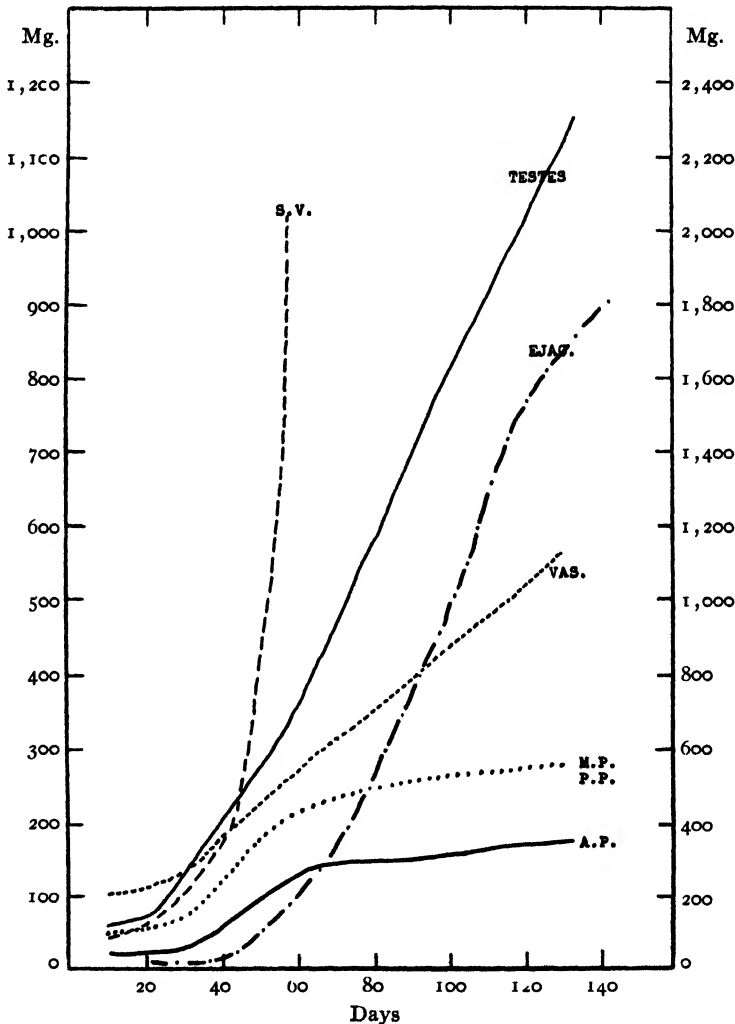


FIG. 1.—Growth curves of reproductive gland weights in normal animals and weights of ejaculates of prepuberal guinea pigs. Curves drawn by inspection from scatter of points of data in Table 1 and from scatter of points of the weights of 244 electrical ejaculates of normal young animals. Testes and ejaculate weights plotted after the right-hand scale; others, after the left-hand scale.

Aside from details of individual growth increments, perhaps the most striking point is the tendency of all organs to show a pronounced acceleration in weight beginning about the thirtieth to fortieth day of life. Testis increases occur slightly earlier than those of the accessory reproductive organs.

Since a detailed comparison of weights of individual accessory organs with general

body-growth shows a decidedly disproportionate growth on the part of the internal organs, one is immediately impressed with the indications of some critical period in the life of the organism at about this period. Testis weight, though increasing at a more rapid rate than general body-growth, exhibits a smoother general growth than the accessory organs; hence, the initiation of, or enhancement of, testis hormone secretion is probably responsible for the rather sudden accelerated growth of these accessory parts.

FUNCTIONAL DEVELOPMENT OF PROSTATE AND SEMINAL VESICLES

Seminal vesicles of most rodents produce a secretion that sets in a firm coagulum when mixed with prostatic secretions, and in the guinea pig the ejaculate forms a coagulated vaginal plug firmly attached to the walls of the vagina. The secretory function of these accessory organs has been shown to be dependent upon the internal secretion of the testes (Camus and Gley, 1896; De Bonis, 1909; Walker, 1910; Moore and Gallagher, 1930).

It was demonstrated by Battelli (1922) that male guinea pigs experience an ejaculation when stimulated on the head by an alternating electric current, and Moore and Gallagher (1930) have employed the technique as a means of studying the influence of testis hormone in the animal. It appeared advisable in this study to utilize the reaction as a means of determining the onset of function of the prostate and seminal vesicles in young animals, which would, in turn, establish at least the period in testis hormone secretion at which the effective threshold of hormone concentration was reached to induce this function.

Fifty-five guinea pigs were stimulated with electric shocks between the ages of 11 and 89 days after birth; and in case the initial stimulation failed to evoke an ejaculation, subsequent stimulations were given at weekly intervals until the first ejaculation was obtained. These stimulations followed the method of Moore and Gallagher and consisted on each occasion of a series of three shocks of 10 seconds each with approximately a minute interval between shocks. For smaller animals an alternating current of 6 volts was sufficiently strong, and graduated doses were utilized until animals of a weight of 350-400 gm. received between 25 and 30 volts. One needle-point electrode was inserted at the base of the skull just dorsal to the foramen magnum, whereas the other blunt terminal was inserted into the mouth. The collected ejaculate was weighed and examined for coagulation and for spermatozoa.

An analysis of the data shows that coagulable ejaculates may be obtained from animals as early as day 21; at this age a male weighing 194 gm. produced a coagulable ejaculate of 10 mg. The majority of males void a coagulable ejaculate by the thirtieth day of life, since 9 of 11 males receiving their first stimulation on day 26 produced coagulable ejaculates; these ejaculates varied from 1 mg. in weight to 150 mg., or an average of 68 mg. One male failed to void an ejaculate until the seventy-fourth day of life, and all stimulations at ages later than this yielded typical coagulable ejaculates. Analysis of the entire data on this point reveals that, among animals receiving stimulations between ages of 21 and 27 days, 45 per cent produced ejaculates; ages 28-34 days, 61 per cent; ages 35-41 days, 71 per cent; ages 42-48 days, 85 per cent; ages 49-55 days, 93 per cent; over 75 days, 100 per cent.

The curve of weight of the ejaculates shown in Figure 1 exhibits a gradual acceleration up to the oldest animal, tested on day 142. The amount of discharge at this time is essentially within the range of normal for adult animals, since Moore and Gallagher

reported 1.93 gm. discharge as the average weight of the second ejaculate; and in the case of the oldest animal here recorded, the weight was 1.75 gm.

Effects of prepuberal castration.—In order to study further the effects of testis hormone on the function of the prostate and seminal vesicles, as evidenced by the electrical ejaculation technique, a series of 15 young males were castrated and subjected to electrical stimulation. With the exception of 2 males, castrated at 5 and 8 days of age, all animals had been subjected to electrical stimulation at least once prior to castration.

Omitting details on individuals, the following results were obtained from this series of animals. (1) All males that had failed to give a coagulable discharge upon electrical stimulation prior to castration failed entirely to produce an ejaculate subsequent to castration; this was true despite stimulations given during several weeks after operation. (2) Animals castrated prior to day 40 failed entirely to deliver an ejaculate subsequent to operation. (3) Animals producing ejaculates prior to operation never again produced an ejaculate equal in amount to that delivered on the day on which castration was performed. (4) All animals failed to produce more than two coagulable ejaculates subsequent to castration, stimulations being administered each seventh day after operation.

Thus the average normal male produces a coagulable ejaculate by 36 days after birth, in an amount of 45 mg.; and the amount increases each week until maturity is established. The castrated animal either fails to produce ejaculates, in the event this function had not already been established before operation, or exhibits immediate marked regression in this function.

APPEARANCE OF SPERMATOOZOA IN THE EJACULATE

The appearance of germ cells in the developing animal may be indicated from histological examination of testes, from smear preparations of an excised epididymis, or from age at first fertile matings. Since an ejaculation induced by the electrical method involves a reaction of all parts of the tract similar to that occurring under normal mating conditions, it was established as a routine to examine all discharges in saline solution in order to determine the age at which spermatozoa are discharged sufficiently mature to exhibit a normal type of motility. It is again to be emphasized that stimulations were given each seventh day after the initial stimulation. No attempt has been made to determine the age at which fertility is established.

Among the 55 young males whose ejaculation history was closely followed, a considerable degree of variability is found in the first discharge of spermatozoa. Fifty-four days was the earliest and 116 days the latest age at which first discharge of spermatozoa occurred; the latter animal had been subjected to fourteen consecutive weekly stimulations before spermatozoa were obtained in the discharge. More than one-half of the males voided spermatozoa by an age of 75 days, and only one reached an age greater than 100 days before spermatozoa capable of motility were obtained.

The data obtained from living healthy animals, and supplemented by a study of fixed materials (in subsequent sections), thus indicate the progression in acquiring normal reproductive functions. The average animal is capable of voiding its first coagulable seminal discharge on day 36. It becomes evident, therefore, that seminal vesicle and prostate function are acquired considerably earlier than the formation of spermatozoa, which occurred on day 54 at the earliest and on day 75 in one-half the males examined. Should one define "puberty" (as distinct from "maturity") as the time at which sperma-

tozoa capable of motility can be found in a typical coagulable seminal discharge, one could say that puberty is attained in 50 per cent of male guinea pigs by day 75 and in practically all males by an age of 115 days. The average animal at puberty will void a coagulable ejaculate of 300-400 mg. and will possess a body weight of 335 gm.

HISTOLOGICAL STUDY OF THE ORGANS OF THE REPRODUCTIVE SYSTEM

The foregoing account of the general growth-trends of various parts of the reproductive system and the study of the time of attainment of the various functions in the living animal have been closely correlated with a study of the histology of the various portions concerned. In this manner one is on somewhat more secure grounds in offering a physiological interpretation of structural conditions found in fixed preparations. The study of a close series of gonads and accessory organs should therefore be of some interest, especially since the tissues have been correlated with known physiological states determined on the living animal.

Testis.—Certain major features of interest in testis differentiation emerge from a close study of the organ from 77 males sacrificed at an age-range of from 2 to 130 days.

Prior to day 20 the testis consists of solid cords containing inactive spermatogonia and indifferent cells. A seminiferous tubule lumen appears in the majority of testes, from day 30 to day 40, and spermatocytes are present. Spermatids appear from day 45 to day 60, and metamorphosing spermatids ("sperm heads" of Moore, 1936) or spermatozoa were present in 1 case out of 13 between days 40-50, in 2 cases out of 10 between days 50-60, in 4 cases out of 5 between days 60-70; spermatozoa were present in the tubules almost invariably subsequent to day 70.

Spermatozoa are to be found in the epididymis shortly after sperm heads or spermatozoa appear in the testis. Although the earliest epididymal smear showing spermatozoa occurred between days 60 and 70, it will be recalled that in one case of electrical stimulation spermatozoa were present in the ejaculate on day 54: this specific animal was not sacrificed until day 62. Spermatozoa were usually present in the epididymis after day 70.

These observations, while showing a fairly rapid progression in the differentiation of germ cells in the testis, reveal that a greater discrepancy occurs in guinea pigs than in rats as to consistency in terms of age of spermatozoan differentiation (see Moore, 1936).

Seminal vesicles.—The seminal vesicles of 23 normal animals sacrificed at periods from 2 to 80 days of age were carefully studied histologically to determine in general the sequence of conditions from nonfunctional to the fully functional organ. This was supplemented by a study of the same organ from 67 animals castrated during this developmental period and sacrificed at varying periods thereafter. Thus, for example, 12 males were castrated between the fifth and eighth day of life and sacrificed periodically from day 15 to day 130. The total range of cases involved castrations at ages 1-73 days with sacrifices thereafter up to an age of 2½ years.

In general appearance the cells of seminal vesicles of animals of 2-6 days of age are tall and columnar, tending in some limited areas to show a pseudo-stratified condition. The nuclei, in general, occupy a basal position; but the general cytoplasm fails to take a counterstain, excepting that the plasmalemma does stand out sharply. A transitional picture appears in animals 10-30 days of age. Many epithelial cells of the seminal vesicle have acquired a state that permits the cytoplasm to stain quite as does that of mature normal vesicles. Subsequent to day 30, the general appearance of the epithelium does not differ materially from the adult condition.

Castration at day 5 does inhibit the development of this general staining reaction of the epithelial cells, since sacrifice at day 45 or later reveals that the cells stain in accordance with the normal pattern of the 5-day-old pig.

Careful measurements of the epithelial height of seminal vesicles of all categories have been made by means of an ocular micrometer, to determine, first, the progression during normal development and, second, whether castration offers a modifying factor in development.

The results of the study of cell height have been somewhat surprising in the lack of any definite trend in effect, either in normal development or from castration. Variations in cell height determined from 23 normal males at ages of 2-80 days presented a range from $17\ \mu$ to $25\ \mu$, or an average of $20\ \mu$. Individual ranges have given no consistent increment in height, since in vesicles from animals 2 days of age cell height was greater than in vesicles removed from animals 60 days of age. The average cell height has therefore been attained by 2 days after birth.

Changes in cell height as a result of castration have been surprisingly inevident and inconsistent. One indication of involution in seminal vesicle epithelium of castrates occurs in cases where castration of short duration only begins at approximately the age of establishment of secretory function. Thus, an animal castrated at day 35 and sacrificed 10-20 days later may show an epithelial height of $11-13\ \mu$, which is lower than any normal male in the series. On the other hand, another animal castrated on the same day but permitted to live for more than 2 years may show an epithelium appreciably higher. Thus, temporary reduction in cell height is suggested, but such reduction fails to characterize clearly a castrate condition.

Golgi apparatus.—The lack of any specific cell inclusion capable of definite diagnosis of the secretory state of the seminal vesicle of guinea pigs, such as portrayed by the large "secretion granules" of rat seminal vesicles by Moore, Hughes, and Gallagher (1930), suggested a study of the general responses of the Golgi body. The above-mentioned authors found definite castration modification in the Golgi bodies of the rat tissues.

The study of the progressive development of the Golgi body in seminal-vesicle epithelium of normal guinea pigs reveals that up to approximately 20 days of age the tall columnar cells contain a few osmicated granules either scattered or clumped in the lumen end of the cells. In slightly older males the cells reveal a more extensive granular state. The granules may be grouped in the lumen end of the cell, or occasionally the cytoplasm peripheral to the nucleus may be quite filled with osmophilic granules. By the forty-fifth day of life the previous copious supply of granules becomes aggregated into a knot approximately $8\ \mu$ in height and composed of a mass of coiled threads, usually running approximately parallel to the long axis of the cell. This condition characterized the cells of postpuberal and mature males.

Castration induces some modification in the differentiation of the Golgi body. In general it may be stated that the stage characteristic of normal males approaching maturity is not attained in animals castrated at an early age. Thus, castration prior to day 8 and sacrifice up to the forty-fifth day of age leave the Golgi apparatus in a juvenile state. The lumen end of the cell contains scattered osmicated granules or aggregates of granules. Animals castrated subsequent to the tenth day of age and permitted to live a year or longer still show reduced Golgi material in the seminal vesicle cells, but it is more typical of postpuberal castrates and may assume the form of a condensed nuclear cap. It becomes apparent that the histological condition of the seminal vesicles of the

guinea pig does not provide an adequate index of its functional state. Although electrical ejaculation proves that the vesicles have a normal function at an early age, there is a lack of clear histological condition to indicate it. There is an absence of cellular inclusions (well-defined granules) or of cell-height increase (or decrease after castration) that provides a reliable criterion for presence or absence of testis hormone.

Anterior prostate gland.—Studies on this portion of the prostate gland have been directed toward progressive differentiation with onset of its normal function and to effects that may be evident from castration; it is to be realized, therefore, that its structure in relation to function has been foremost in the course of these studies.

When cell height of the epithelial lining of anterior prostate acini is considered, one soon realizes that little difference exists between the cells of the inactive gland and those of one fully active. Careful measurements of cell height in glands removed at an age of 2–70 days indicated a range of 16–25 μ , or an average of 21 μ ; greatest cell height occurred in the case of an animal 10 days old, and the lowest in a 15-day-old male.

Certain epithelial changes indicate progressions in development. The acinus lumen of the 2-day anterior prostate was 0.074 mm. in diameter, and this was progressively larger until in a 73-day-old prostate the lumen was 0.59 mm. The youngest anterior prostate epithelium is a simple columnar type. This gradually changes, with approaching maturity, into a pseudo-stratified condition, which changes further into a highly folded type. No characteristic cell inclusion of diagnostic value in determining the onset of active secretion was seen.

Castration at early stages exhibited itself principally by rather slight changes in the character of the epithelium and by an increase in size of the acini. Cell height appears to be unmodified by loss of testes. The epithelium tends to be decidedly less folded, and the cells are usually crowded together, producing a pseudo-stratified or stratified condition.

The osmic-acid technique for Golgi-body studies failed entirely in my hands. In anterior prostates of both young and mature males methods that proved successful with other tissues were unsatisfactory. On the whole, therefore, changes that denoted functional activity on the part of this gland failed to appear in either the progressive development or as a result of castration.

Middle prostate gland.—The middle lobe of the prostate gland does not differ markedly in its reactions from the anterior lobe. There is a progressive development from the columnar epithelial lining of simple acini to the folded epithelium of larger acini. Cell height cannot be closely correlated with age, at least from birth to puberty; and variations from 16 μ to 28 μ yield a general average of 22 μ .

Castration likewise does not markedly affect the histological character of this gland. There is some suggestion of a diminution in epithelial height when the testes are removed about the seventy-fifth day, but so many discrepancies enter that one is incapable of definitely diagnosing castration on the basis of general histological changes. Thus, the middle prostate from an animal castrated on day 35 and sacrificed more than 2 years later consisted of epithelial cells that fell within the range of cell heights in normal males.

Osmic acid impregnation of this portion of the prostate was fully successful despite the somewhat disconcerting results in attempting to relate specific morphological responses with a particular functional state. A definite Golgi body is present in the apical portion of middle lobe cells, and its general morphology in animals a few days old is

quite comparable to that of mature males. The body usually consists of a mass of coiled threads oriented in the long axis of the cell between nucleus and acinus end of the cell. Impregnation appears to be less intense in animals previous to 30 days of age than in mature males.

Golgi-body changes resulting from castration are more definite than is cell-height modifications. Castration between days 23 and 60, with sacrifice 100 days later, induces consistently a reduced Golgi body in the form of a cap against the nucleus of the cell. When operations were done from 2 to 8 days after birth, osmophilic material was present as delicate broken or continuous threads or as scattered granules. This suggests an inhibition of normal development in the Golgi body. The principal effects from castration, therefore, are reduction in size and in intensity of impregnation with osmic acid.

Posterior prostate.—A study of the posterior lobe of the prostate similar to that reported in slightly more detail for the anterior and middle lobes yields results approximating those obtained from a study of those portions of the gland. The lumina of the posterior-lobe acini are fairly characteristic in showing a progression from a diameter of 0.074 mm. in the case of a 2-day-old animal to 0.37 mm. diameter in a 60-day male. Cell height is again too variable to offer a diagnostic criterion of function.

Castration appears to induce a temporary reduction in cell height, but in cases of the longer periods of castration the cells often fall within the range of the normal males. The Golgi-body reactions of long-time castrates are again variable, but there is a decided tendency toward a diminished intensity in impregnation. In general the Golgi-body impregnation is sharpest in this lobe.

Vas deferens.—The vas deferens was studied in progressive sexual differentiation and in castration, but it likewise fails to offer outstanding structural conditions that can be definitely associated with a functional condition. The lumen of the tube increases progressively from 0.18 mm. prior to day 15 to a diameter of 0.40 mm. in 60-day males. The epithelial folds increase gradually in height; the cytoplasm of the cells stains deeply; and the apical ends of the cells are quite irregular.

Castration tends to reduce the diameter of the lumen, but considerable variability exists. No appreciable change in cell height follows removal of the testicle, and diagnosis of secretory states in this structure are difficult from general histological appearances.

Golgi-body reactions do not clearly indicate a secretory state in cells of the vas deferens as sexual differentiation progresses. The body lies in the long axis of the cell, apical to the nucleus. It is entirely similar in cells from the 6-day male and normal adult male. Castrations, particularly when performed at about 45 days of age, induce fairly distinct and consistent reductions in the osmicated materials, since the normal large knotlike mass becomes, in the castrated animal, sparse osmophilic granules scattered in the cytoplasm peripheral to the nucleus, or it may take the form of a thick threadlike structure. It is clearly reduced despite the lack of cell-height reduction.

DISCUSSION

The observations here reported were derived from a study of the progression in post-natal sexual differentiation in the male guinea pig. This study involved: (1) the individual growth of various parts, as exhibited by determinations of fresh weights on

carefully dissected organs; (2) the histological condition from birth to a well-developed functional state; (3) the study of the organs after castration performed at various ages; (4) the utilization of such functional tests as the capacity of spermatozoa to exhibit motility, the ability of seminal vesicles and prostate gland to provide normal secretions making up a coagulated ejaculate, and the ability of the animal to void such an ejaculate when properly stimulated.

Considering first the general growth of the reproductive parts, one is impressed with the sudden growth stimulus which occurs slightly earlier in the testis (at approximately the twentieth day) than in the accessory organs of reproduction (about 30-40 days of age). The curves of growth representing these trends fail, naturally, to portray individual variability; hence, analyses of individuals in respect to various aspects become enlightening. This analysis has been utilized both with respect to germ cell development and with respect to the internal secretory activity of the testes.

One of the most definite indexes of testis hormone secretion in the guinea pig is the voiding of a coagulable ejaculate, since this assures a functional state in both prostate and seminal vesicle, which function is under testis hormone control (see Moore and Gallagher, 1930). The observations reveal a fairly widespread variability in the attainment of this function in individual males. The youngest male to perform an ejaculation following electrical stimulation did so on day 21, whereas the oldest tested animal to first attain this capacity was 74 days of age. Fully one-half of the males delivered small coagulable ejaculates by day 30. This coincides approximately with the period of a rise in the curve of weights of the accessory organs. Thereafter both the growth curve and the curve of weights of ejaculate mounted upward until the early stage of maturity was reached. These responses therefore provide indications of the effectiveness of testis hormone.

The study of the progression in testis differentiation, carried on principally by histological study of the testes, was supplemented both by examining smear preparations of the epididymis and by an examination of the electrically induced seminal discharge for spermatozoa. Some variability again exists; but, on the whole, there is a fair consistency in the rate of germ-cell differentiation for all males studied. Metamorphosing spermatids (sperm heads) were first noted at an age of 48 days. This stage of development was attained by only 1 male among 13 examined between days 40 and 50; 3 among 10 males reached the sperm head or spermatozoan stage of development between days 50 and 60, while the majority of animals examined from 60 to 70 days had attained the same stage. One male only was found in which testes did not show spermatozoa at 70 days or later, and the age of this male was somewhat in question. On the whole, spermatozoa were not present in the epididymis until day 65, though one male voided a few motile spermatozoa in an ejaculation on day 54; motile cells appear in the ejaculate at approximately the same time as found in epididymal smears. In one case, only a few spermatozoa were found in the epididymis on day 73, when the ejaculate induced immediately prior to sacrifice failed to show them.

These considerations indicate a fairly rapid metamorphosis of germ cells, passage into the epididymis, acquisition of capacity for motility, and discharge through an ejaculation. Seventy days after birth represents a stage of development in the male guinea pig when the great majority of animals will void coagulable ejaculates containing spermatozoa that are actively motile.

The microscopical study of the accessory organs of reproduction yielded somewhat

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surprising results in that structural conditions of the various parts change very little with the onset of function in normal progressive differentiation or with the loss of hormone following removal of the testes. A greater rapidity and extent of structural change with modifications in the hormonal state would be expected to be more apparent in this rodent in the light of such outstanding changes in the rat and ground squirrel (see Moore, Price, and Gallagher, 1930; Moore, Hughes, and Gallagher, 1930; Price, 1936; Wells, 1935). Whereas in normal differentiation the general character of the cytoplasm gives some indication of the changing functional conditions, cell height is modified but little. The height of the mature epithelium in many of these organs has already been attained at 2 days of age, and in early or later stages of castration there is only slight modification. Histological detail does not readily permit distinguishing the accessory reproductive organs of a male castrated longer than a year from those of normal males. In no technique employed by me have distinctive or diagnostic cell inclusions been found that were capable of denoting a definite functional state on the part of these accessory glands of reproduction. The condition of the Golgi bodies has most nearly approached this capacity. In certain portions (posterior prostate, and vas deferens) a clearly progressive differentiation was evident; the conditions are more or less characteristic of each tissue studied, and in some of these castration induced fairly evident changes from the normal. In other organs, however, progressive development failed to present apparent differences, and castration often produced such slight changes as to make it almost impossible to diagnose the state as one of lack of function lost through castration. The guinea pig, therefore, is a poor test animal for the study of effects of testis hormone on the histological state of the accessory reproductive organs. The electrical ejaculation test, on the other hand, and possibly also the spermatozoan motility test (not used by me; see Moore and McGee, 1928), is a useful test method.

In a later paper more detailed studies will be reported from the study of the effects of castration in mature animals.

SUMMARY AND CONCLUSIONS

1. Growth-rates of testes and accessory reproductive organs of male guinea pigs are accelerated from days 30-40; thereafter growth is rapid.
2. Ejaculations are induced as early as day 21, and the majority of males demonstrate this capacity by approximately day 30.
3. Spermatozoa first appear in the testis about day 50; they are fairly uniformly present by day 70. Spermatozoa appear in the epididymis and are discharged in an electrically stimulated ejaculate within a few days from their first appearance in the testes.
4. Histological study of accessory organs of reproduction during the age of functional development shows a lack of progression in cell-height increases. Prepubertal castration exerts but little modification in prostate and seminal vesicles. Some tendency for lowered cell height is suggested in castrations subsequent to about day 30. No specific cytoplasmic inclusions of diagnostic value for the process of secretion have been noted. Golgi-body changes provide some evidence of lack of testis hormone but are too variable to be of diagnostic value.

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MOTOR AND SENSORY HYPERPLASIA FOLLOWING LIMB-BUD TRANSPLANTATIONS IN CHICK EMBRYOS¹

(Seven figures)

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THE factors controlling growth and differentiation of the vertebrate nervous system have been extensively studied in amphibian embryos. The experimental analysis revealed three main groups of factors: (1) patterns of local proliferation and differentiation, (2) mutual correlations between the different regions of the central nervous system, and (3) growth-controlling factors residing in the nonnervous peripheral structures, to be innervated (see reviews by Harrison, 1935, and Detwiler, 1936). The present report deals exclusively with the last-mentioned factors.

For *Amblystoma*, Detwiler and others have shown that spinal ganglia react sensitively to a decrease or an increase of their peripheral fields. A reduction of their peripheral area (e.g., by the extirpation of a limb) results in a hypoplasia of approximately 50 per cent, whereas peripheral overloading (e.g., by implanting an additional limb) causes a hyperplasia up to 40 per cent. In both instances the spinal cord and, in particular, its motor area showed no reaction; its cell number was in no way changed by the changes in its peripheral fields. Dürken (1912) and May (1930, 1933), on the other hand, reported positive reactions of the spinal cord of the frog, following the same type of operations: a hypoplasia of the motor column after limb-bud extirpation, and its hyperplasia after implantation of an accessory limb. Results similar to the last mentioned were obtained on chick embryos. A marked hypoplasia of the lateral motor column, in addition to hypoplasia of the corresponding spinal ganglia, was found when wing buds were extirpated in chick embryos incubated 3 days (Shorey, 1909; Hamburger, 1934). The difference in the reaction of the motor cells between *Amblystoma*, on the one hand, and the frog and chick, on the other, was attributed to the absence in the former forms, and the presence in the latter, of a distinct lateral motor horn (see Hamburger, 1934, p. 488).

The mechanism through which the peripheral structures affect processes going on in the central nervous system is still obscure. Considering, for a moment, the reaction of the motor horn in the chick, the only clue we have found is in the following observations: (1) the spinal ganglion and the motor column of the same segment react independently of each other; and (2) the motor hypoplasia is proportional to the amount of loss of muscle tissue. Obviously, we are dealing neither with a growth stimulus transmitted through a reflex arc nor with a mass reaction of the central nervous system, but with a localized, almost point-to-point, stimulation of a certain area of motor neuroblasts by their own end-organs (differentiating muscles). The tentative explanation was proposed that a growth-controlling agent travels in centripetal direction along the pathfinder motor fibers which first reach the peripheral area. This agent would be produced in a quantity proportionate to the size of the peripheral area to be innervated and would control, in a quantitative way, either the mitotic activity or certain steps in the differentiation of the neuroblasts which lie in the neighborhood of the pathfinder neurons (Hamburger, 1934).

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It was hoped to obtain more information on this point by studying the reaction of the motor horn of the chick to peripheral overloading. Its effect must not be necessarily the reverse of the hypoplastic effect following a reduction of the peripheral area. In the case of hypoplasia, one might still be inclined to attribute this growth inhibition to a general, nonspecific factor. On the other hand, a positive effect in the form of a localized growth stimulation of a given region of the motor column beyond its normal size, exerted by an enlargement of its own peripheral field, would strongly support our assumption of a specific growth-controlling mechanism.

THE OPERATION

Our experimental approach was the same as in the respective amphibian experiments; certain levels of the spinal cord were overloaded by implanting a supernumerary limb into their vicinity. The operations were made on chick embryos at 60–70 hours of incubation, i.e., at a time when the first motor fibers begin to emerge from the spinal cord. In these stages the wing and leg buds project as small buds from the flank of the embryo. The donor embryo was removed from the blastoderm, transferred into a watch glass containing 0.9 per cent salt solution, and the wing or leg bud excised with an iridec-tomy knife. The host embryo was then prepared: a window was sawed into the shell; a longitudinal slit was made with a glass needle into the flank near the outer borders of the somites; and the transplant was placed in this slit. It usually became attached to the body wall of the host embryo. The window was closed, and the host embryo was returned to the incubator for a period of 8–9 days. The technique has been described in detail in earlier papers (Hamburger, 1938, 1939).

THE INNERVATION OF THE TRANSPLANTS

Our expectation that host nerves would enter the transplants was fulfilled in many cases. However, it was found that only the transplants located near the spinal cord received an appreciable nerve supply; those located ventrally, or others which were attached to the mesenteries, either remained nerveless or were poorly innervated. The dorsal transplants were supplied by nerves originating in the adjacent levels of the spinal cord of the host, i.e., transplants located near the host wing were supplied by brachial nerves, transplants located near the host legs were supplied by lumbar nerves, whereas transplants located in the trunk region were entered by trunk nerves alone or by a combination of trunk and limb nerves. The number of nerves supplying a transplant was usually smaller than that of a normal wing or leg. Any nerve or nerves that entered a transplant formed a more or less complete and typical nerve pattern within the limb, irrespective of their source of origin and of their quantitative development. These and the following observations are based on reconstructions from serial sections of the innervation of thirty-two transplants (Hamburger, 1939).

THE QUANTITATIVE DEVELOPMENT OF TRANSPLANT NERVES

A comparison of transplant nerves with their partners on the left (unoperated) side showed that they were hyperplastic. However, their increase in diameter was always limited and never very conspicuous. As a result, the ingrowing nerves did not meet fully the increased demand, the innervation of a transplanted limb always remained far below saturation. In only a few cases did we find an increase in nerve diameter above 100 per cent. Since the number of transplant nerves was always smaller than normal, each one would have to enlarge by several hundred per cent if a quantitatively complete in-

nerve were to be accomplished. This growth limitation of the transplant nerves proved to be a serious impediment to the present study. If a considerable enlargement of the peripheral fields causes only a slight increase of the peripheral fibers, then no marked change in the number of neurons can be expected.

TABLE 1
TOTAL CELL COUNTS OF LATERAL MOTOR HORN CELLS

POSITION OF TRANSPLANT	CASE NO.	AGE (DAYS)	TRANSPLANT INNERVATED BY NERVES NUMBER	SECTIONS COUNTED EVERY—	TOTALS	
					Total Number*	Difference
Wing in wing-level					Brachial Swelling	
	tre 419	9	15-17	other	r = 7,217 l = 6,684	533 (8%)
	tre 379	9	16	third	r = 4,106 l = 3,890	216 (5%)
Control	gn1	9	13-16	other	r = 6,740 l = 6,634	106 (1.5%)
Leg in leg-level					Lumbosacral Swelling	
	tre 21	8	21-23	third	r = 5,969 l = 5,120	849 (16.5%)
	tre 393	9	22-24	other	r = 9,112 l = 7,980	1,132 (14.2%)
	tre 347	8	22, 23	third	r = 5,797 l = 5,899	-102 (-1.7%)
	tre 330	8	22, 23	third	r = 5,585 l = 5,545	40 (0.7%)
	tre 340	8	21-24	third	r = 6,246 l = 6,261	-15 (-0.25%)
	tre 355	9	23, 24	third	r = 6,149 l = 6,206	-57 (-0.9%)
	tre 383	9	23, 24	third	r = 5,695 l = 5,671	24 (0.44%)
Control	gn1	9	23-29	other	r = 8,271 l = 8,238	33 (0.4%)

* Symbols: r=right; l=left.

For reasons discussed in the introduction we are particularly interested in the reactions of the motor centers. Unfortunately, motor fibers are more scarce in transplants than are sensory fibers. In several transplants numerous fibers were found to approach and to enter the skin, but only a few entered muscle fibers. In such cases the hyperplasia of the peripheral nerves must be due largely to an increase in sensory fibers. This fact adds to the above-mentioned difficulty in obtaining a demonstrable motor hyperplasia.

TABLE 2

SEGMENTAL CELL COUNTS OF LATERAL MOTOR HORN CELLS IN THE BRACHIAL REGION

CASE No.	THIRTEENTH SEGMENT		FOURTEENTH SEGMENT		FIFTEENTH SEGMENT		SIXTEENTH SEGMENT	
	Total Number	Difference	Total Number	Difference	Total Number	Difference	Total Number	Difference
Tre 419	r = 1,225 l = 1,225	0 (0%)	r = 2,544 l = 2,508	36 (1.4%)	r = 2,375 l = 2,103	273 (13%)	r = 1,073 l = 848	225 (26.5%)
Tre 379	r = 417 l = 437	-20 (-4.6%)	r = 1,306 l = 1,341	-35 (-2.6%)	r = 1,570 l = 1,439	133 (10%)	r = 813 l = 673	140 (20.8%)
Control 9n1	r = 392 l = 430	-38 (-8.9%)	r = 1,916 l = 1,978	-62 (-3%)	r = 2,685 l = 2,639	46 (1.7%)	r = 1,747 l = 1,590	157 (10%)

TABLE 3

SEGMENTAL CELL COUNTS OF LATERAL MOTOR HORN CELLS IN THE LUMBOSACRAL REGION

CASE No.	TWENTY-THIRD SEGMENT		TWENTY-FOURTH SEGMENT		TWENTY-FIFTH SEGMENT		TWENTY-SIXTH SEGMENT	
	Total Number	Difference	Total Number	Difference	Total Number	Difference	Total Number	Difference
Tre 21	r = 462 l = 245	217 (88%)	r = 922 l = 765	167 (22%)	r = 894 l = 804	90 (11%)	r = 900 l = 660	240 (36%)
Tre 393	r = 1,205 l = 1,040	165 (15%)	r = 1,918 l = 1,196	722 (60%)	r = 1,785 l = 1,192	593 (50%)	r = 1,240 l = 1,429	-189 (-13%)
Control 9n1	r = 957 l = 948	9 (1%)	r = 1,543 l = 1,573	-30 (-1.9%)	r = 1,280 l = 1,244	36 (2.8%)	r = 1,342 l = 1,246	96 (7.7%)

CASE No.	TWENTY-SEVENTH SEGMENT		TWENTY-EIGHTH SEGMENT		TWENTY-NINTH SEGMENT		THIRTIETH SEGMENT	
	Total Number	Difference	Total Number	Difference	Total Number	Difference	Total Number	Difference
Tre 21	r = 907 l = 868	49 (5%)	r = 947 l = 847	100 (12%)	r = 569 l = 595	-36 (-4%)	r = 368 l = 336	32 (9%)
Tre 393	r = 1,345 l = 1,231	114 (9%)	r = 779 l = 805	-116 (-15%)	r = 840 l = 997	-137 (-16%)
Control 9n1	r = 1,258 l = 1,310	-52 (-4%)	r = 1,143 l = 1,173	-30 (-2.5%)	r = 748 l = 744	4 (0.5%)

OBSERVATIONS ON MOTOR HYPERPLASIA

The wing- and leg-level of the spinal cord of the chick show large ventrolateral swellings, the lateral motor horns or columns. They include the motor neurons of the limb nerves. Between the lateral motor horn and the floor of the central canal is located the mesial motor column. Its fibers supply the axial musculature; their cell bodies and nuclei are smaller than those of the lateral motor cells. The lateral motor columns are absent in the trunk level between the brachial and the lumbosacral swellings, i.e., in the level of the seventeenth to the twenty-second segment.

We first considered the feasibility of inducing the formation of a lateral horn in the trunk-level by overloading trunk nerves. Transplants located favorably in the dorsal trunk region and supplied purely by trunk nerves were, indeed, obtained. However, their innervation was quantitatively very poor; no large lateral motor neurons were discovered in the respective level of the spinal cord, and not even the mesial motor group showed any appreciable hyperplasia.

It was therefore decided to place the transplant in a position where one of the limb plexuses would contribute one or more nerves to its innervation, so that any reaction would appear as a hyperplasia of an existing lateral motor column. This was done in two ways: a leg bud was transplanted immediately in front of the host leg bud, or a wing bud was transplanted immediately behind the host wing bud. The slits in the somatopleure of the host which were to receive the transplant were extended into the base of the respective host bud. We thus obtained leg transplants which were partly innervated by posterior trunk nerves and partly by anterior lumbosacral nerves 23 and 24 (or exclusively by the latter two), and wing transplants which were innervated exclusively or predominantly by the posterior brachial nerves (15 and 16). Nine such cases were selected for further studies. The innervation of these cases is indicated in Table 1.

Method of cell counting.—The establishment of a hyperplastic effect is based on the comparison of cell numbers of the right (overloaded) and the left side of the spinal cord.

Cross-sections, 10 μ thick and stained with Heidenhain's hematoxylin, were used. The lateral motor neurons can be identified readily by their large size and the dark deep stain of their cell bodies. Their nuclei were counted on both sides on every other or every third section of the entire brachial or lumbosacral swelling. Only nuclei containing nucleoli were counted. The considerably smaller cells scattered in the motor horn, which are presumably glia cells and few in number, were omitted. The nuclei were counted with a Veeder counter and, at the same time, were checked on paper by use of a camera lucida, so that each cell would be counted but once.



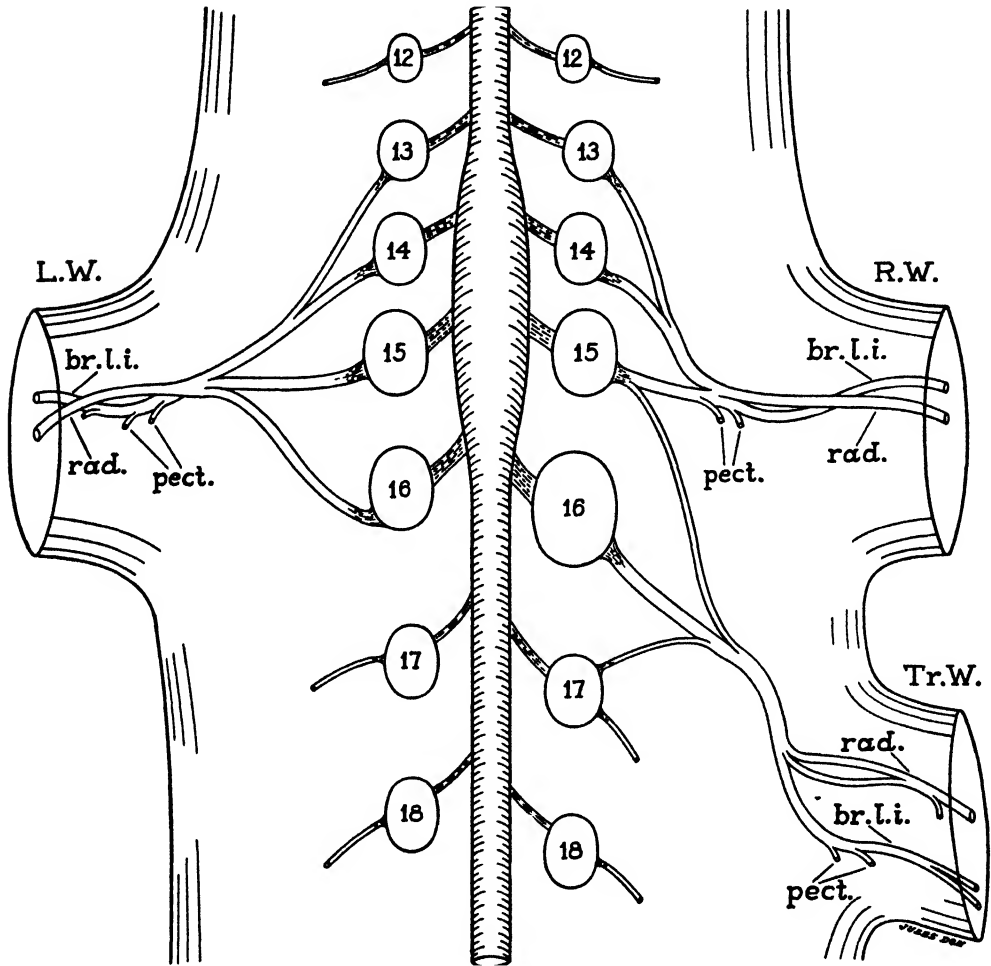
FIG. 1.—Tre 419. Right wing transplanted posterior to right host wing. Age at fixation, 9 days.

Tre 419.—The transplant was developed normally but was slightly smaller in size than the host wing (Fig. 1). It was innervated (Fig. 2) by nerve 16 and small branches of

MOTOR HYPERPLASIA IN THE BRACHIAL SWELLING LEVEL

Two cases were selected in which a transplanted wing was located immediately posterior to, and in the same horizontal level as, the right host wing. Both transplants showed spontaneous motility.

nerves 15 and 17. All three united in a plexus before entering the transplant. They formed a typical wing nerve pattern; its two main nerves—nervus radialis and nervus brachialis longus inferior—and their main branches were traceable. Strong nerves to the



Tre 419

FIG. 2.—Tre 419. Graphic reconstruction of the innervation of the transplant. *L.W.*, left host wing; *R.W.*, right host wing; *Tr.W.*, transplanted wing; *Br.l.i.*, nervus brachialis longus inferior; *Rad.*, nervus radialis; *Pect.*, nervus pectoralis.

pectoral muscles emerged from the nervus brachialis longus inferior of the transplant. Not less than seventeen different nerve bundles were found to enter the muscles of thigh and shank. The innervation of the host wing was accomplished by nerves 13–15.

The right nerve 16 was larger than the left one; the right ganglion 16 was greatly hyperplastic; and the right ganglion 15 was slightly hyperplastic. No appreciable increase in the diameter of peripheral nerve 15 was detected.

The lateral motor neurons were counted in every other section of the brachial swelling. A total difference of 533 cells was found, the hyperplasia of the right side amounting to

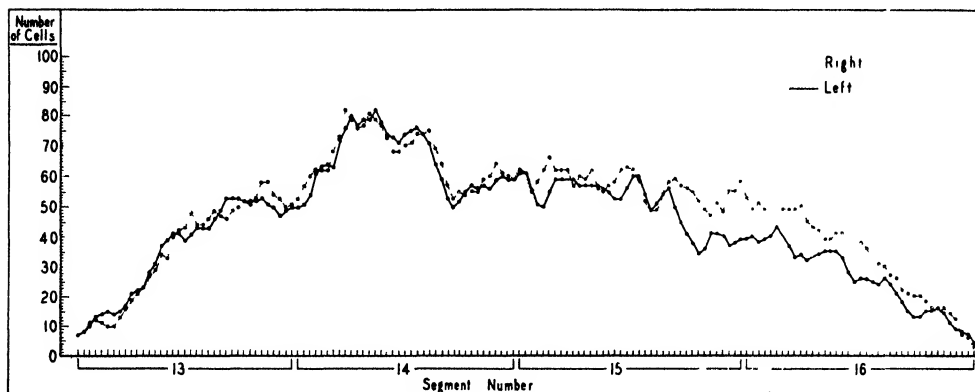


FIG. 3.—Tre 419. Comparison of the numbers of lateral motor neurons in the right and left half of the spinal cord (brachial swelling). Every other section was counted. Each point on the graph represents the average of five consecutive sections.

8 per cent. In order to obtain an accurate picture of the distribution of this hyperplasia over the extent of the brachial level, a graphic representation (Fig. 3) was constructed, in the following way: The ordinate is an estimate of the number of cells per section, and the abscissa the sequence of sections. The boundaries of the segments indicated on the abscissa were obtained by tracing the motor roots of the different nerves. The method of estimating the number of cells per section was to average the cell number in five consecutive counts, then omit the first count, add the next following count, and average these five counts, and so forth.



FIG. 4.—Tre 379. Right wing transplanted posterior to right host wing. Age at fixation, 9 days.

Table 2 shows clearly that no appreciable cell difference exists in segments 13 and 14 and that the hyperplasia begins in segment 15 and is highest in segment 16. In this latter segment the cell numbers of the right side exceed those of the left side almost with no exception. The percentages of hyperplasia, calculated for the four segments separately, are: 0 per cent in segment 13, 1.4 per cent in segment 14, 13 per cent in segment 15, and 26.5 per cent in segment 16 (see Table 1). Obviously, the hyperplasia is strictly localized in the posterior part of segment 15 and in segment 16. The latter supplies the transplant, while segment 15 contributes a branch to the transplant innervation and, in addition, may have to substitute for nerve 16 in the nerve supply of the host wing.

Tre 379.—The transplant (Fig. 4) is a fully developed wing immediately behind the host wing. Its innervation is supplied by nerve 16 (see reconstruction in Hamburger,

1939, Fig. 3). The strong transplant nerve branches into a *nervus radialis* and a *nervus brachialis longus inferior* and forms a typical, though incomplete, pattern in the transplant. The nerve bundles which were found to enter the transplant muscles are less numerous than in the preceding case. The nerves to the pectoral muscles of the transplant are completely missing. We may, therefore, expect a lower degree of hyperplasia than in tre 419. However, the right spinal ganglion 16 is considerably hyperplastic. The lateral motor neurons were counted in every third section. A total hyperplasia of the right horn, amounting to 5.5 per cent was established by the cell counts. This figure would not seem to be significant, although, when the cell numbers were plotted as before, a local hyperplasia in the sixteenth segment, amounting to 20.8 per cent, was detected. Again, the cell figures of the right side are consistently above those of the left side throughout the sixteenth segment.

A normal 9-day embryo was sectioned, and the motor neurons of its brachial region were counted on every other section. The total difference was 1.5 per cent in favor of the right side. The fluctuations within the four brachial segments were: +8.9, -3, +1.7, and +10 per cent, if the left side is taken as the basis. (This method makes the figures comparable to those of the operated cases.) The unaffected segments 13 and 14 of tre 419 and tre 379 can likewise be considered as control segments. We find here differences of 0, +1.4, -2.6, and -4.6. per cent.

Compared with these fluctuations, those of segments 16 in the experimental cases, amounting to 26.5 and 20.8 per cent, respectively, must be considered as significant. The differences in segments 15 are perhaps doubtful. However, the graphs show that these slight increases are located at the posterior ends of the fifteenth segments and mark the beginning of the hyperplasia which is continuous through the sixteenth segment.

We conclude that the motor column of the brachial region can be stimulated to localized hyperplastic growth by peripheral overloading.

HYPERPLASIA IN THE LUMBOSACRAL SWELLING

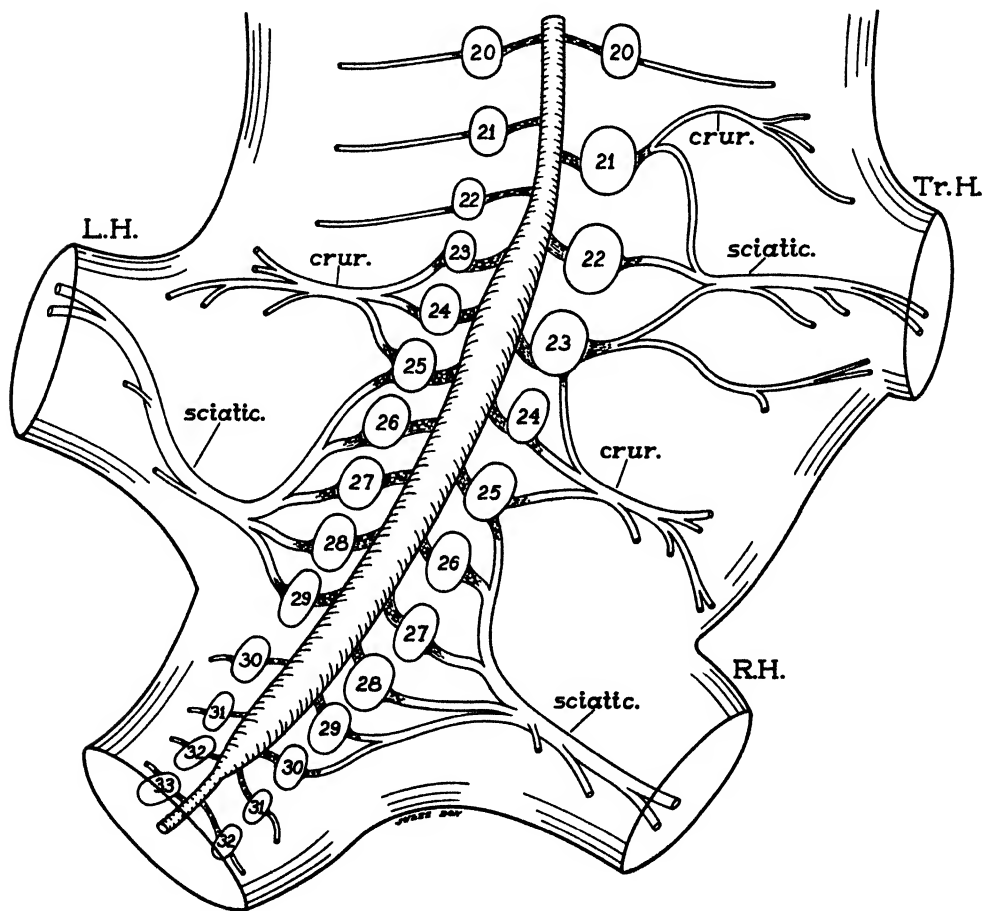
The lumbosacral plexus of the chick (Fig. 5, left side) is formed by spinal nerves 23-29 (sometimes 23-30). Nerves 23, 24, and part of 25 unite in an anterior plexus, which subsequently forms the *nervus cruralis*. The posterior branch of nerve 25, together with nerves 26-29 (30), form a posterior plexus, from which the *nervus ischiadicus* emerges. Transplants located immediately in front of the host leg are usually supplied by a variable combination of posterior trunk nerves (21 and 22) and anterior lumbosacral nerves (23 and 24). Seven such cases whose nervous systems had been reconstructed previously were chosen for cell countings. Their innervation is given in Table 1.

A definite hyperplasia was found in two cases (tre 21 and tre 393); it amounted to 16.5 and 14.2 per cent, respectively. No hyperplasia was found in five cases. We shall take up the positive cases first.

Tre 21.—The transplant, a well-formed leg which showed spontaneous movements, was innervated (Fig. 5) by two trunk nerves, 21 and 22, and the anteriormost leg nerve, 23. Part of the latter, together with the former two, formed a joint plexus from which emerged the sciatic nerve of the host; part of nerve 23 entered the transplant directly and innervated its pelvic region. All these nerves were mixed, but their motor branches were not abundant. The right nerve 30 joins the lumbosacral plexus of the host leg. The total number of ganglia is 40 on either side.

Cell counts of the lateral motor nuclei of the entire lumbosacral swelling show a total difference of 849 cells (16.5 per cent).

In view of our observations on the brachial swelling we would expect to find this hyperplasia localized at the anterior end of the lumbosacral region and to find little, if any, difference in posterior regions.



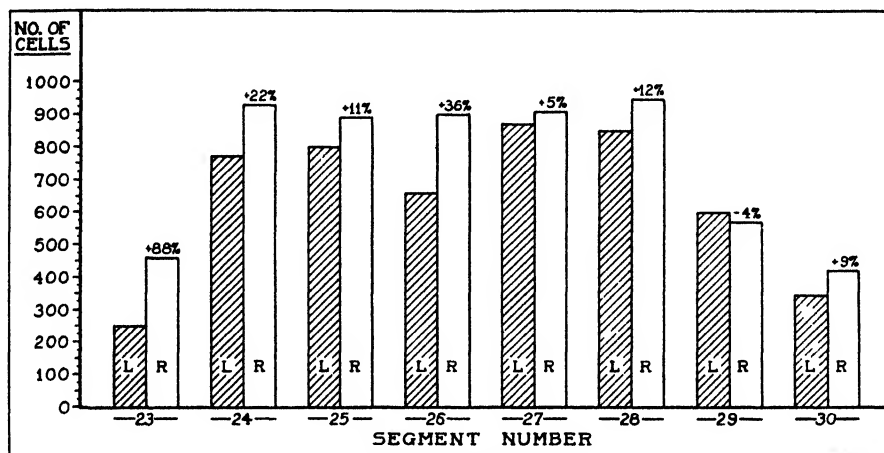
Tre 21

FIG. 5.—Tre 21. Graphic reconstruction of the innervation of the transplant. *L.H.* and *R.H.*, left and right host legs; *Tr.H.*, transplanted leg; *Crur.*, nervus cruralis; *Sciatic.*, nervus ischiadicus.

Unfortunately, the demonstration of a local effect in the lumbosacral level is made very difficult, for the following reason. When a transplant is located in front of the right host leg, the growth and expansion of its base causes a considerable bending of the posterior trunk and tail region of the host. As a result, the spinal cord is distorted, and no symmetrical cross-sections of it can be obtained in the critical level. This curvature of

the spinal cord is indicated in the shift of the spinal ganglia. As it is impossible to compare the left and right side section by section, another, less accurate, method was chosen. The motor roots of all spinal nerves involved were traced to the points of their emergence from the spinal cord. It was thus possible to establish with some approximation the boundaries between two consecutive segments on the left and the right side, separately, and to compare the cell numbers in corresponding left-half and right-half segments. Figure 6 presents the results in a graphical form; the actual figures are given in Table 3. The hyperplasia is most marked in the anterior segments 23 and 24 and in segment 26. The differences in the other segments are considered as doubtful.

The spread of the hyperplasia over other segments than the one which is actually involved in the innervation of the transplant can be interpreted as a compensatory effect;



Tre 21

FIG. 6.—Tre 21. Comparison of the numbers of lateral motor neurons in the right and left half of the lumbar segments of the spinal cord. Every third section was counted.

an alternative explanation is that motor fibers originating in segments 24 and 26 grow cranial into the transplant nerves.

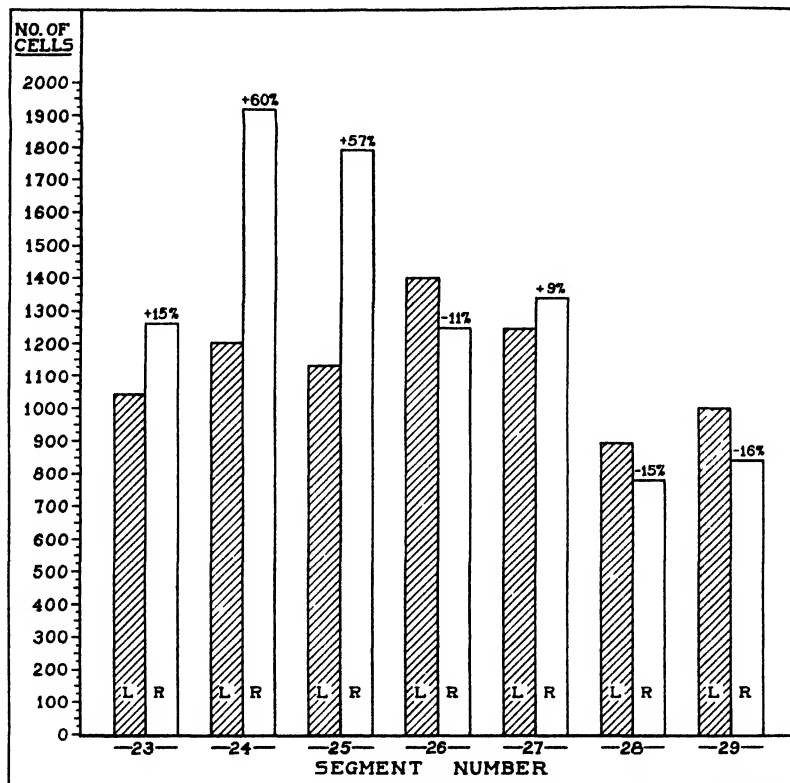
The trunk nerves 21 and 22 are considerably hyperplastic, as are their spinal ganglia. However, no hyperplasia was discovered in their motor centers. The number of mesial motor neurons was found to be equal on both sides (right side, 1,235 cells; left side, 1,238 cells). The hyperplasia of these nerves must therefore be accounted for either by an increase in sensory fibers only or, if the number of motor fibers is also increased, by splitting of peripheral fibers instead of by cellular hyperplasia. A third explanation would be that motor fibers originating in more caudal segments enter these transplant nerves. We favor the first assumption, as the total motor supply of the transplant is not abundant and might well be accounted for by the normal number of motor fibers present in nerves 21–22, increased by the considerable motor hyperplasia in nerve 23.

Tr 393.—The transplant, a normally differentiated leg (see Hamburger, 1939, Fig. 21) which showed spontaneous movements, was located anterior to the right host leg. It was innervated by trunk nerve 22 and by lumbar nerves 23 and 24. Nerve 24 also

contributed a posterior branch to the lumbosacral plexus of the host. Numerous motor fiber bundles were found to enter the transplant muscles.

The nuclei of the lateral motor horns were counted in every other section throughout the entire lumbosacral swelling. The total difference was found to be 14.2 per cent in favor of the operated side.

The trunk region of the host was again strongly bent; therefore, the same method as before was applied to calculate the percentage of hyperplasia per segment (Fig. 7 and



Tre 393

FIG. 7.—Tre 393. Comparison of the numbers of lateral motor neurons in the right and left half of the lumbosacral segments of the spinal cord. Every other section was counted.

Table 3). Again we found considerable differences in the anterior segments 24 and 25 (amounting to 60 and 50 per cent, respectively). The spreading of the hyperplasia over segment 25, which is not directly involved in the innervation of the transplant, may again be explained either as a compensatory effect (nerves 24 and 25 alone form the crural nerve of the host) or by assuming that fibers from this segment grew craniad and entered transplant nerves. Nerve 23, apparently, does not contain an excessive amount of motor fibers.

Cell counts of the lumbosacral swelling of the control embryo on 1 (on every other section) showed a total difference of only 33 cells (0.4 per cent). Since the embryo was

straight, an accurate comparison of left-half and right-half segments was possible. The differences within the individual segments ranged from 0.5 to 7.7 per cent. The differences in the unaffected posterior segments of the operated animals, which may also be considered as control segments, ranged from 4 to 16 per cent. It is obvious that the local differences of 50–88 per cent found in anterior segments of operated animals are significant.

Cell counts were made in five other cases of well-developed leg transplants. None of them showed any hyperplasia, the differences are below 1 per cent in four out of five cases, and in favor of the left (control) side in three cases. The absence of any reaction can be explained in two ways: either the motor innervation of these transplants was very poor, and no motor fibers in excess of those which are normally present in the transplant nerves were formed, or, if the motor supply was considerable, other mechanisms than cellular hyperplasia (e.g., a bifurcation of existing fibers) must have come into play.

Tre 347.—This case can be disposed of without further discussion. No motor hyperplasia was to be expected, as the only transplant nerve which originated in the lumbosacral level of the host (nerve 23) contained exclusively sensory fibers.

In the remaining four cases (*tre 330*, *tre 340*, *tre 355*, *tre 388*) motor fibers entering transplant muscles were exceedingly scarce; their number was considerably smaller than that of the four positive cases. It would seem that the amount of motor fibers normally present in nerves 23 and 24 would be quite sufficient to account for the motor innervation of these transplants, and no hyperplasia would be expected. Although the possibility of a peripheral bifurcation cannot be ruled out definitely, the cases under discussion certainly do not necessitate or even warrant such an assumption.

SENSORY HYPERPLASIA

It had been shown previously (Hamburger, 1934) that in the chick, as in amphibians (Detwiler, 1920), a decrease in the peripheral sensory area results in a marked hypoplasia of the corresponding ganglia. The present experiments show that an increase in peripheral area results invariably in a definite hyperplasia of the ganglia supplying the supernumerary limbs.

The following observations are based on a material of thirty-two sectioned and reconstructed cases.

In studying the peripheral distribution of the transplant nerves it was found that the sensory innervation, though by no means abundant, was more complete than the motor innervation. In accordance with this observation we found a hyperplasia in the spinal ganglion of almost every nerve which entered a transplant. Even slight increases in the size of transplant nerves were reflected in an enlargement of their ganglia.

Our method of reconstruction of the nervous system permits detection of even slight differences in the length of left and right ganglia. In order to obtain quantitative data, paper models of a selected number of ganglia were prepared and their weights compared, following a method applied by Detwiler (1920) and others. The complete sequence of sections of a given pair of left and right ganglia was outlined on cardboard with the camera lucida, cut out, and weighed. Data thus obtained give a rough estimate of the increase in volume (see Table 4). Every transplant ganglion shows an increase in volume with the exception of *tre 419*, ganglion 17. The branch from nerve 17 to this transplant is very small. The hyperplasia is highly variable, ranging from 15 per cent (which figure

may not be significant) to over 200 per cent. We suspect that this variation is quantitatively correlated with the variation in the increase of the peripheral fields, but no attempt was made to verify this point.

The table shows that brachial, lumbosacral, and trunk nerves react equally well. It brings out a further point of interest: the localization of the effect. With few exceptions only those ganglia responded which were actually involved in the supply of the transplant, whereas neighboring ganglia remained unaffected. Of the exceptions to this rule, some do not seem to be significant (e.g., tre 379, ganglia 14 and 15; tre 393, ganglion 21). The only case of a conspicuous hyperplasia of over 40 per cent in a ganglion not innervating a transplant is tre 383, ganglion 25. A plausible explanation of its excessive growth is that of a compensatory effect. This ganglion is the only one remaining of the three crural

TABLE 4

COMPARISON OF WEIGHTS OF PAPER MODELS OF SPINAL GANGLIA (IN GRAMS)

TRANS- PLANT No.	TRANS- PLANT INNER- VATED BY SPINAL NERVES NUM- BER	GANGLION NUMBER														
		Brachial					Trunk					Lumbosacral				
		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Tre 419	15-17	r=6 l=5.2	r=4.9 l=2.9	r=1.8 l=1.8	r=2 l=2
Tre 379	16	r=1.2 l=1.2	r=2.5 l=2.2	r=3.2 l=2.8	r=2.6 l=1.9	r=1.1 l=1.1
Tre 510	13-16	r=1.9 l=1.3	r=4.7 l=3.6	r=5 l=4.4	r=3.9 l=3.1
Tre 346	21-23	r=1.1 l=0.9	r=1.1 l=1.1	r=1.4 l=0.9	r=1.9 l=1.1	r=1.7 l=1.5
Tre 383	23, 24	r=1.5 l=1.1	r=3.2 l=1.2	r=2.4 l=2.1	r=1.7 l=1.2	r=3.7 l=3.5
Tre 393	22-24	r=1.4 l=1.2	r=1.8 l=1.3	r=2.7 l=2.4	r=3.0 l=1.2	r=1.9 l=2.1	r=2.7 l=3	r=2.4 l=2.2

ganglia of the host, nerves 23 and 24 being involved in the innervation of the transplant. A hyperplasia is to be expected if ganglion 25 alone, as the reconstruction shows, is burdened with the formation of the crural nerve of the host. Several other cases can be easily explained in the same way.

We conclude that the reactions of the sensory centers, like those of the motor centers, are more or less strictly localized in the levels which are actually overloaded peripherally. We are, obviously, not dealing with a nonspecific mass action of the outgrowing transplant tissue on the spinal cord. Additional convincing evidence ruling out this alternative is given in the following case. Transplant tre 347 is innervated by nerves 20, 22, and 23, whereas nerve 21, incidentally, did not enter the transplant. Although all four ganglia developed at approximately the same distance from the outgrowing transplant tissue, only the ones which were actually overloaded showed a hyperplasia, whereas ganglion 21 remained unchanged. Another case presents the same feature.

DISCUSSION

An attempt was made to produce a hyperplastic effect in the lateral motor column of the brachial swelling of the spinal cord by implanting an additional wing near the host wing, and in the lumbosacral swelling by implanting a leg near the host leg. Cell counts demonstrated a hyperplasia in four out of nine cases (two in the brachial, two in the lumbosacral region). The percentages of hyperplasia, if calculated for the entire brachial or lumbosacral swellings, were low, ranging from 5.5 to 16.5 per cent. However, when the percentage hyperplasia was calculated for individual segments of the spinal cord, then the hyperplastic effect was found to be localized in few segments, where it ranged from 13 to 88 per cent (average for eight affected segments, 37 per cent), whereas other segments remained practically unaffected.

It is of importance to notice that the hyperplastic segments were those from which the overloaded transplant nerves emerged, whereas the unchanged segments were those which did not contribute to the transplant innervation. The most striking illustration of this point was found in the two cases of brachial hyperplasia, where a point-to-point comparison between the left and the right side of the spinal cord was possible (Fig. 3). Here we found that in the overloaded posterior segments the cell numbers of the right side were constantly above those of the left side, whereas they were practically identical, section by section, in the unaffected anterior segments. A spreading of the hyperplastic effect over segments which were not overloaded but were immediately adjacent to "transplant" segments was suggested as a compensatory effect.

This intimate correlation cannot be incidental. We consider these data as additional evidence for a direct growth stimulation of local motor centers in the spinal cord by the peripheral muscle areas which these centers innervate. As was pointed out in the beginning of the article, the simplest and most satisfactory explanation of the mechanism involved here would be to assume a growth-controlling agent traveling in centripetal direction from the periphery along the first motor fibers to the growing motor centers. Alternative explanations—e.g., the transmission of the stimulus via a reflex arc, or a general growth reaction of a certain level of the spinal cord to adjacent, rapidly growing transplant tissue—are not supported by the facts. The former possibility is made unlikely by the independent size variations of spinal ganglia and motor columns in the same segment; and a mass reaction is practically ruled out by the observation that only segments which actually innervate the transplant show hyperplastic effects, whereas neighboring segments which are not overloaded but which are close to the transplant do not, in general, show any effect. A corresponding situation was found in experiments on wing extirpation (Hamburger, 1934).

A similar correlation exists between the increase in peripheral sensory fields and the growth of the corresponding spinal ganglia.

The five negative cases do not invalidate this conclusion but find a satisfactory explanation in the observation that these transplants had received an extremely poor motor innervation or none at all.

Is the increase in cellular proliferation in the positive cases sufficient to account for the excess of motor fibers found in the transplant? Or is it necessary to assume that some of the additional motor fibers were produced by a peripheral splitting of axons? This mechanism of fiber increase has been described for amphibians (Weiss, 1931, 1937; Detwiler, 1933, p. 290). In the best-analyzed case (Weiss, 1937) it was found that if in old

larvae of *Amblystoma* one brachial nerve was cut and introduced into an implanted accessory limb, the subsequent hyperplasia of this transplant nerve was accomplished by a bifurcation of peripheral nerves and not by a cellular increase in the motor center.

No quantitative data are available in our material to compare the central, cellular hyperplasia with the peripheral, fibrillar increase, but it seems that they are approximately in agreement. The amount of accessory motor fibers present even in the best-innervated cases was small enough to be readily accounted for by the respective cellular hyperplasia. Our material does not, therefore, necessitate the assumption of peripheral bifurcation.

The surprisingly small amount of hyperplasia which is obviously related to the quantitatively poor nerve supply of the transplants deserves a brief comment. As was pointed out before (Hamburger, 1939), the pathfinder fibers succeeded in most cases to locate the transplant, but these first connections failed to receive an adequate reinforcement.

Why this failure? Wing-bud extirpations have resulted in a hypoplasia of up to 60 per cent of the total number of motor neurons. One is therefore inclined to attribute to a normal limb a considerable growth-stimulating activity. The failure of a transplanted limb to manifest such an activity at a corresponding order of magnitude may be due either to a central or to a peripheral inhibition. Either the potency of the spinal cord to produce an excessive number of motor neurons is limited or, if this potency is not restricted, the stimuli exerted by the transplant are weaker than those originating in a normal limb. The greater distance between the transplant and the spinal cord may play a role in many cases. The time factor must also be considered. The exploring pathfinder fibers may find the transplant in an advanced stage of differentiation, a fact which may make establishment of further connections and the transmission of centripetal stimuli more difficult.

COMPARISON WITH AMPHIBIA

Numerous experiments on Amphibia have shown that spinal ganglia and other sensory centers react sensitively by hyperplastic growth to an increase in the size of their peripheral sensory fields (review in Detwiler, 1936). In this respect the data on amphibians and on the chick are in full agreement. However, marked differences between different forms were found when the effects of peripheral changes on motor centers were tested. Detwiler (1924 and later) has given convincing evidence that the extirpation of one or even of both forelimb primordia, in *Amblystoma*, does not result in a decrease of the number of motor neurons in the corresponding segments of the spinal cord, the only perceptible effect being a slight size reduction of individual neurons (Detwiler and Lewis, 1925). On the other hand, Dürken (1912) and May (1930, 1933), using different genera of anurans, have described a motor hypoplasia following limb-bud extirpation. Similarly, wing-bud extirpations in 3-day chick embryos resulted in a marked hypoplasia of the lateral motor horn (Hamburger, 1934).

Corresponding discrepancies were discovered when the response of the motor center to peripheral overloading was studied. Detwiler summarizes his experiences with the urodele *Amblystoma* as follows: "When one, two or even three limbs are grafted to a heterotopic position and are innervated chiefly by the 6th and 7th nerves, the ventral motor areas of these nerves do not show any numerical increase in response to the added musculature" (1933, p. 290). On the other hand, May (1933), working on the anuran *Discoglossus*, described hyperplastic reaction of the spinal cord following the implanta-

tion of a hind-limb bud in a position near the normal hind limb. The present investigation again places the chick in line with anurans and in contrast to urodeles.

May's conclusions are based on cell counts in three of his five cases. He found an excess of cells on the operated side, amounting to 8-33 per cent in the segments innervating the transplant. His data are not directly comparable to my figures, as he counted all cells in the ventral half of the cross-section; and he counted only ten sections per segment. In one case, in which four segments adjacent to the "transplant" segments were counted as a control, three of these showed a hyperplasia of the right side of almost the same amount as the "transplant" segment. His data are altogether inadequate to permit any conclusion as to the actual amount and the localization of the hyperplasia.

The differences in the reaction of motor centers between anurans and the chick, on the one side, and urodeles, on the other, are obviously real, since they are demonstrated in experiments of overloading as well as reduction of peripheral fields. We have suggested that they might be due to structural differences in the spinal cord, the former types possessing a definite lateral motor column, which is missing in the more primitive cord of *Amblystoma* (Hamburger, 1934).

SUMMARY

1. Wing primordia of a 3-day chick embryo were implanted immediately posterior to the right wing bud of a host embryo of the same stage. They became innervated by posterior brachial nerves. Cell counts of the lateral motor neurons of the brachial region in two cases showed a motor hyperplasia on the operated side which was localized in the segments innervating the transplant and which amounted to 26.5 and 20.8 per cent, respectively, in the affected segments.

2. Hind-limb primordia were implanted immediately anterior to the leg buds of host embryos and received part of their innervation from the anterior lumbosacral nerves of the host. Cell counts of lateral motor neurons in the entire lumbosacral swelling showed, in two cases, a total hyperplasia of the right side amounting to 16.5 and 14.2 per cent, respectively. A precise determination of the localization is difficult for technical reasons; but the available data indicate that, again, the hyperplasia was localized in the segments which actually contributed to the transplant innervation. It amounted to between 20 and 88 per cent in the affected segments.

3. The spinal ganglia of transplant nerves showed a definite hyperplasia.

4. These data give further evidence that in the chick embryo the peripheral fields control the growth of both sensory and motor centers in the spinal cord.

POSTSCRIPT

In a recent publication, Yü-Ch'üan Tsang (*Jour. Comp. Neurol.*, 70 [1939], 1) showed that in a strain of unilaterally polydactylous mice the retrodorsolateral column of the motor horn was hyperplastic in twelve out of nineteen cases. This coincidence of a strictly localized motor hyperplasia and of a localized peripheral hyperplasia is in full agreement with the data reported above, and suggests that in mammals the same causal relation exists between peripheral growth and nervous growth as in the frog and the chick. Tsang considers the growth abnormality of the central nervous system as the primary cause of polydactyly. This interpretation is untenable. His own negative results in seven of nineteen cases demonstrate that hyperdactyly can occur in the absence of changes of the central nervous system. Furthermore, we have shown that limbs of frog and of chick embryos will develop normally in the complete absence of any innervation (see Hamburger, 1939). Finally, the present paper demonstrates directly that a motor hyperplasia can be produced by an enlargement of the peripheral area to be innervated.

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THE PRODUCTION OF DUPLICITAS CRUCIATA AND MULTIPLE HEADS BY REGENERATION IN EUPLANARIA TIGRINA

(One plate and five text figures)

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THESE experiments are based on a modification of an operation first reported by J. van Duyne in 1896. He produced a duplication in planaria resembling the duplicitas cruciata which has been described for other animals, such as the salamander (Spemann, 1919, Wessel, 1926, Wittmann, 1929), the frog (Penners and Schleip, 1928), the scorpion (Brauer, 1917), *Tubifer* (Penners, 1924), and *Chaetopterus*

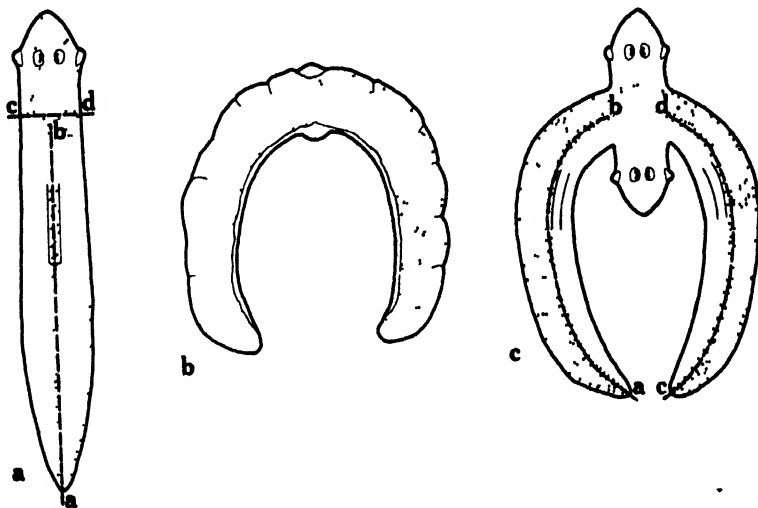


FIG. 1. (a) Operation in Experiment 1, (b) 3 days after operation (note simultaneous appearance of anterior and posterior heads), (c) 8 days after operation (a, b and c-d indicate operation in Expt. 2). Clear areas indicate regenerated parts.

(Titlebaum, 1928). Duplicitas cruciata is characterized by two heads in opposite directions, separated by two tails also opposite each other and with their median plane perpendicular to that of the heads.

Van Duyne produced these duplications by making a single longitudinal cut through the median line of the planarian extending anteriorly to the head region. In a few cases in which the half-tails did not grow back together, a second head formed posteriorly at the anterior end of the slit. This operation has been repeated with occasional success by Morgan (1900), Bardeen (1901), Goetsch (1921, 1922), Keil (1924), and Beissenhirtz (1928).

We modified this operation in the following manner: a longitudinal cut was made through body and tail to a point halfway between the auricles and the pharynx (Fig. 1, a [a-b]), and then the head was removed by a transverse cut that left two half-tails

held together at their anterior ends by a narrow connection approximately one-third their width ($c-d$). The removal of the head permits the half-tails to assume a more outstretched condition and eliminates motion to a great extent. Both of these effects tend to produce a high percentage of successful cases. Beissenhirtz (1928) used the same modification. Duplications of the posterior heads were observed occasionally, but none occurred at the anterior cut surface.

In a second experiment the half-tails were allowed to regenerate for 8 days and were then split again in their new median planes. Here again supernumerary heads developed; but head development was distinctly lateral in most cases, in contrast to the crotch position found in the first experiment. The origin of such supernumerary heads was analyzed further in a third experiment.

Euplanaria tigrina (syn. *Planaria maculata*) was used in this experiment. The animals were collected from the pond behind the Episcopal church in Falmouth, Massachusetts (near Woods Hole), during August and early September, 1938. They were always used within 1 day after collection and were not fed. They were kept in tap water at room temperature.

Animals 11–14 mm. in length were placed on a glass plate in a few drops of water and were cut under a low-power binocular microscope by means of a piece of razor blade glued to the flattened end of a 5-inch piece of glass tubing. Narcotics were not used, since they have been shown to influence head frequency.

EXPERIMENT I

In this experiment 100 animals (in groups of about 25 each) were cut, as shown in Figure 1, *a* ($a-b$). The pharynx was removed. After 24 hours the cut surface had healed over and the two half-tails had fused back together in varying degree. They were split with a needle to reopen the old longitudinal cut; and at the same time the head was removed by a transverse cut, leaving two half-tails held together by a narrow strip at their anterior cut ends (Fig. 1, *a* [$c-d$]). Thus, the transverse cut was delayed 24 hours.

About 10–15 minutes after the transverse cut, the animals became immobile in an outstretched condition. The two tails assumed a horseshoe shape, with their median longitudinal surfaces completely exposed and facing posteriorly and with their anterior surface only partially exposed, owing to extension of the half-tails and contraction of the edges. This combination of tail extension and contraction of the anterior cut surface brings the edges of the latter together and results in their partial fusion. The anterior cut surface thus becomes reduced in size (Fig. 2, $a-d$). In a few cases fusion was complete and no anterior surface was exposed (Fig. 2, *d*).

Since we found that the amount of anteriorly exposed tissue is correlated with the type of regeneration at the anterior end, we separated the animals 24 hours after the second cut into two groups: those with relatively much wound surface exposed anteriorly, and those with little or none.

Some individual cases were protocolled every day, but the majority were examined in groups of about 5–15 at least five times during the first 8 days after the operation. Final data were compiled on the eighth day after the transverse cut was made.

RESULTS

After the outstretched condition described previously had been assumed, the cut surfaces healed over smoothly—usually within the first day. Any minor irregularities on

the cut surface were completely smoothed over by the formation of new unpigmented tissue.

Distinct, unpigmented outgrowths appeared perpendicular to the anterior and posterior cut surfaces on the third or fourth day after the transverse cut had been made. Eyes became visible about 1 day later. Generally there was little or no visible difference in time of appearance of heads at the two surfaces (Fig. 1, b).

Regenerated pharynges were never observed earlier than the fourth day after the operation, and they did not fully differentiate for about 2-3 weeks. They originated close to the healed surface, that is, almost entirely in the regenerated tissue. Only after

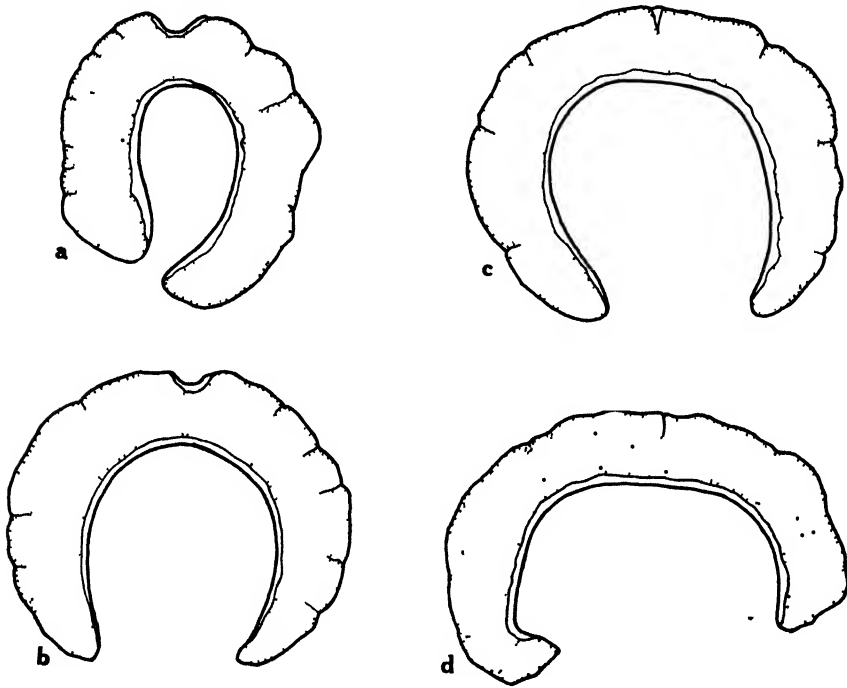


FIG. 2—Variation in extent of fusion at the anterior cut surface 1 day after the transverse cut was made in Experiment 1

lateral regeneration of the tail was complete, after several weeks, did the pharynges take up a median position in the tails. Double pharynges were found in several cases. Beissenhirtz (1928) also observed the lateral origin and duplication of pharynges.

Thus these processes may be said to occur more or less in the following sequence: healing, head formation, development of pharynges, and lateral regeneration of the tails.

After the heads differentiated, either head could take the lead, with the other head and two tails trailing behind. The heads alternated in guiding the movement, which was always either in an anterior or a posterior direction—never was the movement oblique or lateral.

Table 1 summarizes the results of Experiment 1. Anterior and posterior heads on the same animal were obtained in 79 per cent of the cases, anterior heads only, in 12 per

cent; and posterior heads only, in 9 per cent. At the anterior surface single heads developed in all cases (except the 9 cases in which no head formed), but duplicated heads formed posteriorly in 17 per cent of the cases. Abnormal heads were found anteriorly in 9 per cent, posteriorly in 21 per cent. Only 2 cases had both heads abnormal. These heads can be classified in Child's terminology (Child, 1915, p. 107). We found anophthalmic, teratomorphic, and teratophthalmic types.

*Anterior heads absent (Group 3 of Table 1; Fig. 9).—*The absence of anterior heads observed in 9 cases was definitely correlated with the amount of new tissue exposed anteriorly on the day after the transverse cut. An anterior head was entirely lacking when the edges of the anterior wound surface fused together so closely that, after healing, very little or no tissue was exposed (Fig. 2, *d*).

*Anterior heads abnormal (Group 1, *d*, *e*, and *f*).—*The 9 cases of this group, when observed on the day after the second cut, had less tissue exposed anteriorly than usual but more than the 9 cases mentioned in the preceding paragraph (Fig. 2, *c*). As expected, they developed abnormal anterior heads, either with two eyes, one eye, or no

TABLE 1
SUMMARY OF RESULTS OF EXPERIMENT 1

Total cases.....	100
1. Anterior and posterior heads.....	..	79	
<i>a</i>) Anterior normal, posterior normal.....	38		
<i>b</i>) Anterior normal, posterior abnormal.....	19		
<i>c</i>) Anterior normal, posterior duplicated.....	13		
<i>d</i>) Anterior abnormal, posterior normal.....	6		
<i>e</i>) Anterior abnormal, posterior abnormal.....	2		
<i>f</i>) Anterior abnormal, posterior duplicated.....	1		
2. Anterior heads normal, posterior heads lacking..	..	12	
3. Anterior heads lacking, posterior heads present..	..	9	
<i>a</i>) Posterior, single (and normal).....	6		
<i>b</i>) Posterior, duplicated.....	3		

eyes, and no auricles in any case. At the time the data were taken (8 days), 5 of the 9 had no eyes, 3 had single eyes, and 1 had two eyes. (Some animals listed at this time as anophthalmic, however, were found to be teratomorphic, i.e., with single eyes, when a second inspection was made several weeks later.) It is obvious that incidental contraction and fusion is a major factor in determining the type of regenerate in these cases.

*Anterior heads normal (Groups 1, *a*, *b*, *c*, and 2).—*In 82 cases normal anterior heads developed. Of these, 77 had a considerable amount of tissue exposed on the day after the transverse cut (Fig. 2, *a* and *b*); the remaining 5 cases were doubtful when protocolled.

*Posterior heads absent (Group 2).—*Eight of the 12 cases of this category showed a partial fusion of the tails along the longitudinal cut surfaces. The other 4 were doubtful in this respect. Incidentally, all 12 cases belonged to that group which had a large amount of anterior surface exposed on the day after the second cut. It seems that their tails were less outstretched and more nearly parallel than usual (Fig. 2, *a*). This condition, of course, was more favorable for longitudinal fusion (or was caused by fusion), and at the same time prevented the usual decrease in anterior wound surface, owing to the bending and contracting that accompanies the assumption of the horseshoe shape. The

coincidence of large anterior surface and the absence of posterior heads can thus be explained on a simple mechanical basis.

Posterior heads abnormal (Group 1, b and e).—The group of 19 animals (Group 1, b) which developed normal anterior and abnormal posterior heads had much anterior surface exposed like Group 2. Thirteen of these had single posterior outgrowths without eyes or auricles—anophthalmic heads. In a few cases eyes were found imbedded in the tissue at the base of the outgrowth when whole mounts were prepared after several weeks. Either the cases diagnosed as anophthalmic were actually teratomorphic or the imbedded eye appeared later. The latter possibility is more likely—at least for a majority of these animals. The other 6 cases were teratomorphic heads.

Individual cases of these types were seen to develop the posterior protuberance at the same time as the anterior outgrowth, but then the anterior continued to develop a normal head while the posterior was inhibited.

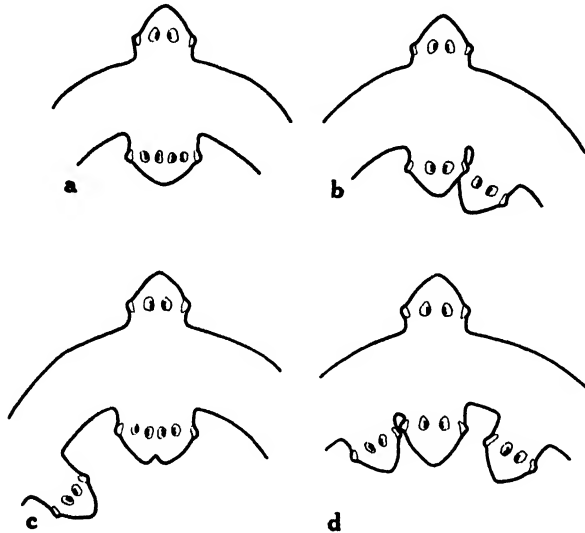


FIG. 3.—Types of posterior duplications found in Experiment 1

Only 2 cases (Group 1, e) were found in which both anterior and posterior heads were abnormal. The anterior heads were anophthalmic, and the posterior were teratomorphic.

Posterior heads normal or duplicated (Groups 1, a, c, d, f, and 3, a, b; Figs. 6-10).—The interesting feature of this category was the appearance of duplications in 17 of the 67 cases in the group. Two or three distinct heads were found in 10 cases. The remaining 7 cases of duplication were more or less abnormal single or double outgrowths with supernumerary eyes. One of the most extreme cases had nine distinct eyes in two outgrowths.

The duplications were almost invariably crowded toward the crotch, which was always occupied by at least one outgrowth. One exceptional case is shown in Figures 3, c, and 10, in which one of two outgrowths was distinctly lateral and far removed from the

second, which was in the crotch as usual. The same specimen incidentally had four pharynges.

Following is a complete list of the duplications:

- 7 cases with 1 outgrowth and 3 or 4 eyes (Fig. 3, *a*)
- 3 cases with 2 outgrowths and 3 or 4 eyes (Fig. 3, *b*)
- 4 cases with 2 outgrowths and 5-9 eyes (Fig. 3, *c*)
- 3 cases with 3 outgrowths and 5-6 eyes (Fig. 3, *d*)

Beissenhirtz (1928) and Morgan (1900) obtained occasional cases of duplications.

Concerning the symmetry of the duplications, we found that in the 10 cases of head duplication one head was always in the crotch and the other or others were lateral to it. The 7 cases of partial head duplications with supernumerary eyes were always in the same plane of symmetry as the anterior head.

EXPERIMENT 2

In an attempt to compare crotch head formation of normal tails and of regenerating tails 20 cases of *duplicata cruciata* were split again. Eight days after the transverse cut of the previous operation, a cut was extended through the tail and trunk to the head region (Fig. 1, *c*). The heads were not removed. The four half-tails were then kept apart by splitting with a needle within a 2-day period. Some cases, nevertheless, healed together again, and others were torn in the process of splitting. These were discarded. (The same experiment was attempted by Beissenhirtz, with little success.)

The cuts were made in the mid-line, which was located at the edge of the heavily pigmented or old tissue; and the regenerating pharynges were removed. Thus, two half-tails, for convenience designated "tail 1" and "tail 4," were composed entirely of old tissue; and the other two, tails 2 and 3, were composed almost entirely of regenerated, unpigmented tissue with a thin strip of old tissue at the cut surface. The old main nerve cords were presumably in tails 1 and 4.

After the operation, the four half-tails twisted around each other violently but eventually assumed an outstretched condition, similar to that assumed by the cases of experiment one. As mentioned above, splitting with a needle was necessary in most cases to keep the four half-tails from healing together; but seldom were they so treated more than once, never more than twice. Since the heads were not removed, these animals were kept in the dark, to eliminate as much motion as possible.

Head outgrowths appeared about 3 days after splitting (Fig. 1, *b*). The half-tails regenerated laterally, and small pharynges became visible in the regenerated tissue soon after the heads appeared, but never earlier than the fourth day.

The most striking features of these animals, as observed within 9 days after the operation, were the following:

1. Contrary to expectation, a large majority of the cases had no crotch heads but developed lateral heads on tails 1 and 4 (Figs. 11 and 12). Thus, of the thirty-one heads that developed, twenty-four (77 per cent) grew out laterally from tails 1 and 4 and seven (23 per cent) developed in the crotch or at least so close to it that they could not be readily distinguished from true crotch heads. Only those that were clearly not in the crotch were classified as "lateral." One case developed two new crotch heads, and thus became a radially symmetrical monster with four heads and four tails.

2. There were no lateral heads on tails 2 and 3.

3. The lateral heads, with the tails from which they developed, tore off from the rest of the animal after they had differentiated.

4. In 3 cases head duplication was observed—i.e., 1 case developed two lateral heads on tail 1, and 2 cases developed both a crotch head and a lateral head on the same cut surface. Five other cases had lateral heads with supernumerary eyes.

In connection with these duplications two specimens obtained in preliminary experiments at Cold Spring Harbor are of special interest. The species was not verified but was probably *Tigrina*. Both were cases of duplicitas cruciata that were split after 8 days like the animals of Experiment 2 (Fig. 1, c).

Case A (Fig. 4) was remarkable. Tails 1 and 4 developed lateral heads, and tails 2 and 3 did not, as in Experiment 2. However, tail 4 developed three lateral heads with two eyes each. Two of the three heads were fused. Tail 1 developed a broad lateral outgrowth along its regenerating surface, with six distinct eyes.

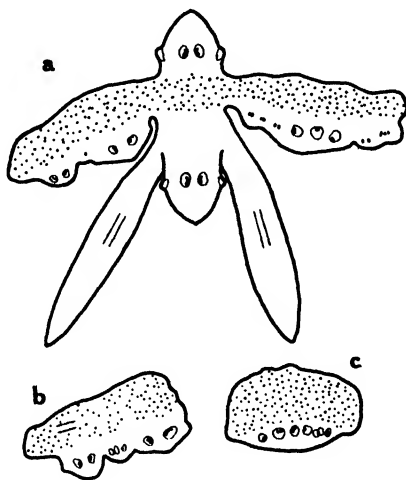


FIG. 4.—(a) Experiment 2: Cold Spring Harbor, case A, 6 days after operation as shown in Figure 1, c, lateral outgrowths on tails 1 and 4; (b) tail 4 after fission, 12 days after operation; (c) tail 1 after fission, 8 days after operation.

Case B developed only a single lateral head on tail 4; but tail 1, which was removed as a half-tail at the time of operation, developed nine distinct eyes in one broad lateral head outgrowth. Again tails 2 and 3 remained headless.

All four tails 1 and 4 described above tore off within 9 days, and three of them developed single pharynges no matter how many eyes or heads were present. The fourth, tail 1 of Case A, was lost.

The results of Experiment 2 are in contrast to those of Experiment 1, where there were relatively few lateral heads and a high percentage of crotch heads and where lateral heads were found only in addition to crotch heads. The difference might be due to the fact that the tails of the second experiment were partly composed of newly regenerated tissue when cut.

EXPERIMENT 3

This consideration led to the third experiment. If the tails of the *duplicitas cruciata* were allowed to differentiate longer, then they should more closely resemble the tails of an unoperated animal. Thus, 12 animals were allowed to differentiate for 16 days and were then cut in the same way as in Experiment 2. By this time the lateral regeneration was almost complete and the pharynges had taken up a position nearer the median line. Pigment had appeared in the regenerated tail tissue, although it was still not as heavy as in the older half of the tail. The behavior and development of these animals after operation was similar to that of the animals in Experiment 2. The results are summarized in Table 2.

TABLE 2
POSITION OF REGENERATED HEADS IN EXPERIMENTS 2 AND 3

	No. of Animals*	Total No. of Regenerated Heads	Lateral Heads on Tails 1 and 4	Lateral Heads on Tails 2 and 3	Crotch Heads
Expt. 2.	17	31	24	0	7
Expt. 3.	12	20	4	1	15

* In 6 cases two of the four tail parts fused back together.

As expected, the ratio of lateral heads to crotch heads was reduced, from 24:7 (in Expt. 2) to 5:15 (in Expt. 3), and approached the results of Experiment 1. The only essential difference was the occurrence in 4 cases of lateral heads in the absence of crotch heads, which was never observed in Experiment 1. We suspect that if differentiation had proceeded farther—that is, if the second longitudinal cut had been delayed more than 16 days—crotch heads would have developed even in these specimens.

One specimen requires special note. It had one lateral head on tail 1 and one on tail 3. This was the only case in which tail 2 or 3 developed a lateral head.

DISCUSSION

THE INTERPRETATION OF THE DUPLICATIONS AS *DUPLICITAS CRUCIATA*

"*Duplicitas cruciata*" is defined as a complete duplication in which the two heads, facing in opposite directions, have a common median plane of symmetry, and where the two tails likewise are opposite each other, with their median plane perpendicular to that of the heads. Such duplications have been described for *Tubifex* (Penners, 1924), *Chaetopterus* (Titlebaum, 1928), *Euscorpius* (Brauer, 1917), *Triton* (Spemann, 1919; Wessel, 1926; Wittmann, 1929), and *Rana* (Penners and Schleip, 1928). Some duplications obtained by transplantation in planaria (Miller, 1938) resemble *duplicitas cruciata*.

Those animals in our experiment which have one anterior and one posterior head fit the foregoing definition. In all these cases the posterior head was directly opposite the anterior. This position of the heads directly opposite each other was visible from the time of the earliest appearance of the head outgrowths (about 3 days).

The position of the nervous system was established by Bessenhirtz (1928). He showed that the nerve cords in the regenerated tissue are connected with the posterior head and that the anterior head is connected with the two old nerve cords. The position of the

four main nerve cords is indicated by dotted lines in Figure 5, *b*. The anterior parts of the intestinal trunks are continuous in the region between the heads and are bilaterally symmetrical (Fig. 8).

Observations of the movements of the animals after their heads had differentiated support our interpretation. Either head could take the lead, with the other head and tails trailing behind. In the same specimen neither head showed continual "dominance," but the heads alternated in directing the movements. No case was observed in which oblique or lateral movement occurred. The animals invariably moved either in an anterior or a posterior direction. All these observations confirm the interpretation that the duplication is a *duplicitas cruciata*.

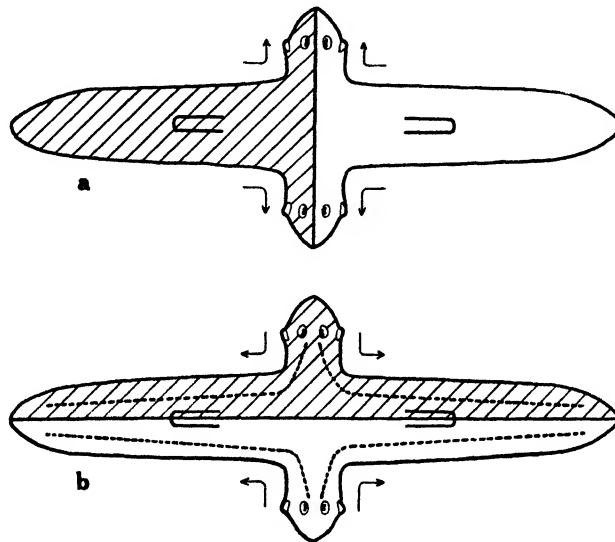


FIG 5. — Diagrams indicating two types of *duplicitas cruciata*: (*a*) Spemann type; (*b*) Schultze type. The dotted lines in *b* indicate the position of the main nerve cords.

According to Keil (1924), the posterior head would be the product of regeneration of one tail, and the anterior head would form a functional and structural unit with the other tail. Our observations rule out this interpretation.

Although all cases of *duplicitas cruciata* in the different animal groups have close geometrical resemblance, an analysis of their origin has made it necessary to distinguish between two different types.

The *duplicitas cruciata* represents a peculiar union of two individuals, in which either the heads or the posterior parts are of a composite nature—i.e., they are shared by both individuals. Schleip (1929) calls the forms with composite ("secondary") heads and uniform ("primary") tails the "Spemann type" (Fig. 5, *a*). The forms with "primary" heads and "secondary" tails (Fig. 5, *b*) he calls the "Schultze type," each type named after the investigator who first produced it experimentally in amphibians.

The two types differ in their embryonic origin. In the Spemann type, differentiation starts from two separate posterior centers instead of one. Differentiation proceeds in both along the same axis. The anterior ends of the primordial individuals meet and

deflect each other in such a way that half of each head primordium now differentiates perpendicularly to the original axis (see arrows in Fig. 5, *a*) and unites with the adjacent half-head of the other individual. The duplicitas cruciata that Spemann (1919) produced by fusing half-gastrulae in *Triton* and the duplicitas cruciata in *Tubifex* (Penners, 1924) and *Chaetopterus* (Titlebaum, 1928) belong to this type.

In the Schultze type, differentiation starts either from two separate anterior (scorpion, Brauer, 1917; arrows in Fig. 5, *b*) or from middle trunk centers (*Rana*, Penners and Schleip, 1928) and proceeds in either case to produce undisturbed primary heads and deflected secondary tails.

The present case is unique in that it does not have embryonic origin but results from regeneration of one individual.

To which type should this case be compared? In regenerating planarians a secondary head would have to be defined as one which has two sources; it would be composed of two "half-heads," each of which is the product of regeneration of one half-tail. If it were technically possible to vital-stain the regenerating cells of one half-tail, the regenerated head would be composed of one stained and one unstained half (Fig. 5, *a*). This interpretation has been advanced by Morgan (1900), Goetsch (1921, 1922), and Beissenhirtz (1928). The following considerations lead us to a different interpretation.

The anterior head cannot be considered a "secondary head." It originated from a single cut surface and never showed evidence of duplication. In fact, the end result can be obtained without removal of the anterior head.

The nature of the posterior head is difficult to establish. In every case of single posterior heads (71 of the 100 cases) the head appeared at the cephalic end of the posterior cut surface directly opposite the anterior head. The regeneration bud was single, i.e., showed no indication that it may have originated from two separate centers lateral to the plane of symmetry.

On the other hand, the duplications (17 cases) might indicate a composite nature of the posterior heads. The significance of head duplications in this connection is weakened by the fact that one head is usually in the crotch (i.e., in the plane of symmetry) and the other (or others) are more laterally located in an asymmetrical arrangement. We would certainly favor the secondary nature of the posterior heads if all the duplications were symmetrical with reference to the head axis. This, however, is rarely the case. In general the evidence favors the primary nature of the posterior heads—an origin from a single source rather than from two separate sources.

If one wishes to complete the comparison of this duplicitas with the Schultze type, one would have to consider the trunks and tails as secondary. This interpretation is favored by the relationship of the main nerve cords to the heads: the two cords of each tail are connected with two different heads.

Observations on contraction in the living animal several weeks after the operation demonstrate the same point. When contractions occur in the two tails and trunks, they take place simultaneously and in the same degree in both. Never is one tail contracted while the other is extended. This is true when either head is leading.

To summarize, this duplicitas cruciata resembles the Schultze type with primary heads and secondary tails.

This interpretation is in contrast to the interpretations offered by previous authors. Goetsch (1921, 1922) and Beissenhirtz (1928) consider the anterior head, as well as the posterior head, as secondary, each half of the posterior head regenerating from one half-

tail (i.e., the Spemann type, Fig. 5, *a*; see also p. 293). Their interpretation is based on the concept that the splitting produces two half-individuals which tend to reorganize by lateral regeneration like a planarian completely split through the median line.

This concept requires the rather strange implication that the anterior head, be it intact or regenerated, becomes "physiologically split" as a result of the individuation tendencies of the trunks and tails, so that eventually one half of the one head and the corresponding half of the other, together with the trunk and tail between them, constitute a new individual.

This assumption of "physiological splitting" under the influence of individuation tendencies set up by the posterior halves is contrary to Child's well-founded concept that a head dominates and thus individuates the parts posterior to it, and not vice versa. Our reasons for considering the posterior head primary have already been discussed.

Thus the interpretation of this *duplicitas cruciata* as the Schultze type with primary heads and secondary tails fits our observations best.

"BIPOLARITY" IN DUPLICITAS CRUCIATA

Bipolar heads have been frequently described in planaria (Morgan, 1898; Rustia, 1925; Watanabe, 1935; Miller, 1937; and others). They are usually produced from very short transverse pieces. The highest frequencies are obtained by operating at post-pharyngeal levels and by the application of a chemical agent, such as chloretone or strychnine.

In our experiment the two heads are certainly bipolar in the true sense of the word; their anteroposterior axes are in the same line but in opposite directions. However, the conditions which result in bipolarity are different in the two types of experiments. In transverse pieces the posterior head arises from a transverse cut surface, which would ordinarily be expected to regenerate a tail. This implies reversal of polarity near the posterior end, which may be a reversal of polarity in the ultra-structure or, more likely, a reversal of an activity gradient.

In the case of *duplicitas cruciata* no such reversal takes place. Although the point of origin of the posterior head is "posterior" to the anterior head and in this sense might be expected to form a tail, it is, at the same time, the most cephalic point of the longitudinal cut surface. This fact and possibly other local factors (to be discussed below) obviously provide such highly favorable conditions for head formation in the crotch that a head develops there in spite of the proximity of anterior tissue. Once head formation is determined at this point, the regeneration bud will grow out in a direction perpendicular to the cut surface. This is, incidentally, opposite in position and direction to the anterior head. In other words, this bipolarity does not make it necessary to assume a reversal of existing polarity.

In this respect we agree with Morgan, Goetsch, and others, who likewise did not consider the posterior head as a polar heteromorphosis.

FACTORS IN THE DETERMINATION AND LOCALIZATION OF HEADS

The previous discussion indicates our point of view that the problem presented by the *duplicitas cruciata* is not one of reversal of polarity or a problem of regulation on the part of two half-individuals connected at their anterior ends, but is a problem of head determination.

What factors are responsible for the origin and types of heads found in our experiments?

We shall discuss the following factors: (1) condition of the wound surface, (2) correlations between the two wound surfaces, (3) gradient systems, (4) nervous system, and (5) local factors determining lateral heads.

1. It has been pointed out previously (p. 288) that the incidence and type of anterior heads depends on the *amount of new tissue exposed* after the healing process. When the half-tails contract in such a way that the anterior surface can fuse completely, no head forms. The subnormal heads (anophthalmic, teratomorphic, etc.) are definitely correlated with partial fusion, which reduces the amount of newly healed tissue (Fig. 2, c). Subnormal or absent posterior heads can be explained similarly as a result of fusion at the crotch (p. 288). Thus, it seems that in our cases the total or partial inhibition of head formation can be adequately explained by the absence or paucity of wound surface.

Therefore, we would expect that, if fusion is complete at one cut surface, the other would be maximally outstretched and thus completely exposed. Indeed, whenever no head developed at the anterior surface, a normal head developed in the crotch, and vice versa (Table 1, Groups 2 and 3).

The importance of the wound surface in the formation of heads in cases of this type is further borne out by the results of a simple modification of Experiment 1. Completely headless, two-tailed animals can be produced in a high percentage of cases by delaying the transverse cut for at least a week. During this time the longitudinal cut surface has healed and partly regenerated. The anterior cut surface fuses completely, as in Group 3 of Experiment 1.

2. Does the development of either head exert *inhibitory or stimulating effects* on the other? Table 1 shows that this is not the case. In 50 per cent of all cases in Experiment 1, at least one normal head was present at each surface. The various combinations of normal, subnormal, and missing heads found in the other 50 per cent do not suggest any significant correlations. This is further borne out by the fact that a crotch head can be produced even when the anterior head is not removed. The occurrence of duplications at the longitudinal cut surface cannot be due to stimulation by the anterior head because 3 cases have developed in the absence of an anterior head and 1 case in the presence of a subnormal anterior head.

In this connection, a preliminary experiment with 4 animals was made in which the anterior surface was increased by a modification of the transverse cut in the first experiment. Instead of a single cut made directly through the animal, two oblique cuts were made, leaving the anterior end of the animal V-shaped with the point anterior. This prevents the anterior surfaces of the two half-tails from fusing and inevitably produces an unusually large amount of anteriorly exposed wound surface. These animals, nevertheless, developed both anterior and posterior heads. These observations do not support the idea of an inhibitory effect of a large anterior surface on the formation of a posterior head.

3. According to Child's theory as applied to planaria, a head always forms at the highest point of a *gradient system*. The *relative* position of indifferent regenerative material in such a system (and not *special* head-forming cells or head-inducing or head-localizing agents at the cut surface) determines the formation of a head from the regeneration bud. "As regards *relations* to the rest of the piece, the reconstitution of a head appears to be primarily a *self-differentiation*" (Beyer and Child, 1930, p. 358).

The head, which is the first structure to regenerate, soon assumes "physiological dominance" or inductive properties over the structures which regenerate later, and in some instances also over regulative processes in the old tissue.

In planaria an anteroposterior and a mediolateral gradient have been established by Child and his co-workers. In our experiment the crotch head is the anteriormost point on the longitudinal cut surface and at the same time is in the median plane; it would therefore be a point of very high activity both in the anteroposterior and in the mediolateral gradient systems, and thus a preferential locus for head formation. It remains to be explained why a crotch head develops so close to the anterior, which might be expected to exert "physiological dominance" over this region on account of its more anterior position. One would have to assume that the crotch is so highly stimulated that it acquires a physiological level equal to that of the anterior cut surface (or of the intact head in the original experiment of van Duyne). In this respect the explanation is similar to that given by Child for polar heteromorphoses in short transverse pieces (Child, 1915, pp. 98 f.).

On the basis of gradient systems it is difficult to account for the single and multiple lateral heads found in association with crotch heads in Experiment 1 (see also van Duyne, Pl. 10, Fig. 4, and Beissenhirtz, Fig. 18) and, usually, in the absence of crotch heads in Experiments 2 and 3. If the crotch head is the highest point on the longitudinal cut surface, why should a head appear at any other point on this surface? It is true that many of these lateral heads develop near the crotch, but in several cases the heads were formed at a much more posterior level (see Pl. I, Figs. 10 and 11; also Beissenhirtz, Fig. 18). In the 2 cases obtained at Cold Spring Harbor, head formation was activated along most of the longitudinal cut surface (Fig. 4).

An explanation on the basis of gradients would make it necessary to assume alterations or (in the Cold Spring Harbor cases) practically complete obliteration of the anteroposterior gradient along the longitudinal cut surface. It is unlikely that such a change should occur as a result of one median cut. At any rate, it would be an exceptional disturbance of the gradient system, for lateral heads are infrequent in our experiments of Type 1 and have never been reported in experiments in which planarians were split into two separate longitudinal halves.

The experiments of Morgan (1898) and of Beyer and Child (1930) in which lateral heads did regenerate from median surfaces perpendicularly to the main axis cannot be compared with our cases, because these investigators studied head formation in very short lateral pieces. In these short pieces with three cut surfaces lying close together, "... conditions at the anterior and the median cut surfaces are apparently so nearly balanced that in some cases the one, in some cases the other, becomes the determining factor" (Beyer and Child, 1930, p. 360). A corresponding explanation cannot be applied to our experiment because the cutting in the median plane exposes two long surfaces which are in continuity at the crotch and at no point disturbed by a transverse incision.

Therefore, we are led to believe that factors other than gradients come into play in determining the formation of these aberrant lateral heads. Such factors must be sought in the structures of the cut surface.

4. Of the structures exposed at the cut surfaces, the *nervous system* has frequently been regarded as a factor in head determination. In *E. tigrina* (*P. maculata*), which we have used in our experiments, this question has been investigated by Child and co-workers (notably Beyer and Child, 1930; Watanabe, 1935). These investigators came to

the conclusion that the nervous system plays a double role in head localization. When the main cords are cut twice, the posterior section of the cords releases an inhibiting agent which travels anteriorly, reduces activities at the anterior cut surface, and thus lowers the head frequency.

On the other hand, it has been suggested that the central ends of main nerve cords or of lateral branches exert a stimulating effect on head formation. This action would be nonspecific in the sense that the exposed nerve cord helps to maintain or accentuates the high activity of the cells of the cut surface and thus assists in creating a "preferential locus for head formation" (Beyer and Child, 1930).

In our experiments the inhibitory effect cannot come into play at the anterior cut surface because the main nerve cords are cut at the anterior surface only. No main nerve cords are exposed at the longitudinal surface.

A stimulating effect by the nervous system may be involved, however. The anterior heads originate in the presence of cut ends of the main nerve cords, which are otherwise undisturbed. The posterior (crotch) heads develop at a surface where strong lateral branches undoubtedly are exposed. The localization of lateral heads might be correlated with the exposure of other lateral branches or with concentration of nervous tissue due to muscular contraction. A careful study of sections at the time of origin of crotch heads and lateral heads would give information on this point.

5. The role of the nervous system in the *localization of lateral heads* is hypothetical in the absence of crucial experimental evidence, and at present we can offer no other explanation for their appearance. However, Experiment 2 is of particular interest in this respect, in that it throws light on certain special conditions which favor lateral head formation.

It was found that lateral heads appeared much more frequently, and usually in the absence of crotch heads, when a longitudinal cut was made through tails which were in the process of regeneration. When ordinary tails were split (Expt. 1), 10 out of 100 cases developed lateral heads in addition to crotch heads; but when these half-tails were allowed to regenerate for 8 days and were then split again longitudinally (Expt. 2), 24 out of 28 formed lateral heads. It will be remembered that the second cut was made in the old, pigmented tissue but very near the site of the original cut. The slight deviation of the plane of cutting between the first and second cuts probably is not responsible for the difference in the results. When the tail was allowed to differentiate further (16 days, Expt. 3) and then cut in the same plane as in Experiment 2, the original ratio of lateral to crotch heads was approximated, 5 out of 20 cases forming lateral heads.

Strangely enough, in Experiment 2, no such lateral heads appeared on tails 2 and 3, which consisted almost entirely of newly regenerated tissue (Fig. 4). It seems as though the presence of the regenerating tissue "conditions" the old tissue for lateral head formation, whereas neither old tissue alone nor regenerating tissue alone shows this property to any extent.

It would be premature to attempt an explanation of this situation. However, it should be pointed out that the plane of the second cut in Experiment 2 coincides with the border between old and new tissue. This level is probably more complex in its structural configuration than the adjacent parts of the body, for it is the gateway for regenerative material migrating in a transverse direction—it is the region where the old and the regenerating organ systems (nervous system, intestine, etc.) make connections, etc. It is conceivable that at certain points of this level there exist local conditions which are

particularly favorable for head formation and that these latent potencies may be activated by the second cut.

A further analysis will have to show to what extent the gradient systems, the nervous system, and other local structural or nonstructural conditions are concerned in the formation of lateral heads.¹

SUMMARY

1. One hundred specimens of *E. tigrina* were split in the median plane to a point between the auricles and the anterior end of the pharynx, and the head was removed by a transverse cut in front of the crotch. In 79 cases regeneration resulted in the formation of two heads, one at the anterior surface and one in the crotch, in addition to the lateral regeneration of the two half-tails. Arguments are presented for the interpretation of this duplication as the Schultze type of duplicitas cruciata.

2. Occasional absence or subnormal development of heads was correlated with contraction or fusion of the cut surface, which resulted in a lack of exposed tissue.

3. In 10 cases, one or two lateral heads formed in addition to the crotch head; in 7 cases, oversized heads with three or four eyes formed in the crotch.

4. When the trunks and tails of the duplicitas cruciata were split again, 8 days after the first operation, the percentage of lateral heads was greatly increased and that of crotch heads was decreased.

5. The lateral heads developed only on the half-tails which consisted of old tissue and never on the half-tails which consisted mainly of regenerated tissue.

6. When the trunks and tails of the duplicitas cruciata were split 16 days after the first operation, crotch-head formation was again predominant, as in the first experiment.

7. The role of gradient systems, of the nervous system, and of other possible factors involved in the localization of heads are discussed.

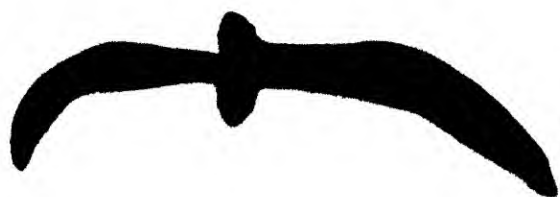
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¹ F. M. Hull (*Anat. Rec.*, 72 [1938], Suppl., 86) reports crotch and lateral head formation in similar experiments. His results seem to be in general agreement with ours.

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PLATE I



1



7



8



9



10



11



12



13

PLATE I

FIGS. 6-7 and 10-13.—Photographs of animals fixed in Zenker's solution and stained with Ehrlich's hematoxylin.

FIG. 6.—Experiment 1: *duplicitas cruciata* several weeks after the operation of Experiment 1. 5×.

FIG. 7.—Same. Double pharynx on right side. 5×.

FIG. 8.—Experiment 1: *duplicitas cruciata*, 16 days after operation. Animal fed a mixture of trypan blue and egg yolk. Note lateral position of pharynges as a landmark for the regenerating side of the tails. 8×.

FIG. 9.—Experiment 1: 16 days after operation, anterior head absent. 8×.

FIG. 10.—Experiment 1: *duplicitas cruciata*, 8 days after operation. Duplication of crotch head, formation of a lateral head, and duplication of both pharynges. 8×.

FIG. 11.—Experiment 2: 10 days after operation as shown in Figure 1, *c*. Formation of two lateral heads on tail 1. 8×.

FIG. 12.—Experiment 2: 6 days after operation; one anterior head, two crotch heads, and one head slightly lateral (its lateral position was unmistakable on the living animal). 8×.

FIG. 13.—Experiment 3: 8 days after operation; one anterior head, two normal crotch heads, and one subnormal crotch head. 8×.

RESPIRATORY METABOLISM DURING LARVAL AND PUPAL
DEVELOPMENT OF THE FEMALE HONEYBEE
(*APIS MELLIFICA* L.)

(Five figures)

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THE present study is an investigation of the respiratory exchange accompanying the differentiation of female larvae of the honeybee (*Apis mellifica* L.) into queens and workers. The honeybee affords an unusual opportunity for measuring the respiratory metabolism of a larval insect, as honeybee larvae are relatively inactive. Insects, although able to live anaerobically for a short time, derive their energy from oxidation. Therefore, the respiratory exchange of insects is of significance since it provides an index to the rate of metabolism. The ratio between the carbon dioxide production and oxygen consumption is known as the "respiratory quotient" (R.Q.), and, as Richardson (1929) has stated, it "is the most direct measure, so far devised, of the relative quantity of foodstuffs entering into the metabolism of the human or animal organism."

The results obtained by Melampy, Willis, and McGregor (unpublished) from a biochemical study of the growth of the reproductive and worker castes of the female honeybee indicate that differences in metabolic activity are associated with caste differentiation. The queen, or reproductive caste, is characterized in the larval stage by a more rapid growth as well as by a higher nitrogen, lipid, reducing substance, and energy content, as compared with the worker caste. Since the beginning of the nineteenth century, biologists have suggested that the difference in nutrition of the castes determines whether a female larva of the honeybee will develop into a queen or a worker. During the first three days of larval life the female larvae receive royal jelly. After this period the reproductive caste continues to receive this diet, whereas that of the worker caste is altered.

The writers are indebted to Dr. T. C. Evans, of the State University of Iowa, and Dr. E. J. Boell, of Yale University, for their helpful suggestions.

REARING OF INSECTS

From eight to ten colonies of Italian bees were used to produce the workers for this study. In order to have available larvae and pupae of a known age, a queen was caged on a worker brood comb. After 24 hours the queen was removed, and the comb containing the eggs was dated and placed in the brood nest. Further oviposition on numbered combs was prevented by excluding the queens.

Since the queen was caged for 24 hours on the brood frame, it is apparent that there would be a 24-hour age range for the larvae. In Tables 1, 2, and 3 the limits of age range are presented; for example, the 0-1 entry under "Age (Days)" in Table 1 means that the oldest larvae would be 1 day old and the youngest would have just hatched.

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The queens were produced by transferring newly hatched female larvae to queen cells and allowing the larvae to be reared in queenless colonies.

The average live weight per individual insect was obtained by weighing representative groups of insects at 24-hour intervals during larval and pupal development. The insects were weighed in groups of 25 to 150 individuals, depending upon the stage of development. This was necessary since it was observed that considerable variation existed between the chronological age and physiological and morphological development. Each value in Table 1 represents the average weight of at least 450 individuals and not more than 900.

The results presented in Table 1 for the growth of the larval worker honeybee are in agreement with the data for the same caste published by Nelson, Sturtevant, and Lineburg (1924), except that the average weight at the 4-5-day stage is lower.

TABLE 1

AVERAGE WEIGHTS OF QUEEN AND WORKER HONEYBEES AT 24-HOUR INTERVALS
DURING THE LARVAL AND PUPAL STAGES

Age (Days)	Live Weight of Queens (Milligrams)	Live Weight of Workers (Milligrams)	Age (Days)	Live Weight of Queens (Milligrams)	Live Weight of Workers (Milligrams)
0-1.....	0 20	10-11.....	243.47	135 63
1-2.....	0 00	1.12	11-12.....	215 38	134.53
2-3.....	10 55	6 08	12-13.....	195 54	131.31
3-4.....	44 93	25.38	13-14.....	124.42
4-5.....	133 24	96.64	14-15.....	126 67
5-6.....	256.56	143 61	15-16.....	126.12
6-7.....	262 34	136 89	16-17.....	124 16
7-8.....	240.70	135 56	17-18.....	122.71
8-9.....	240 60	134.21	18-19.....	113.36
9-10.....	242 05	130 65			

RESPIRATORY MEASUREMENTS

The queen emerges as an adult in approximately 13 days after the hatching of the larva, and the worker in 18 days. For respiratory measurements the queens were selected up to 11-12 days of development, and the workers up to 16-17 days.

The Barcroft-Warburg technique was used to obtain the data on oxygen consumption and carbon dioxide production. All measurements were made on individual insects at $35^{\circ} \pm 0.02^{\circ}$ C. A larva or pupa was placed in the well of the Warburg flask, and 10 per cent potassium hydroxide or water was placed in the main chamber, according to whether oxygen consumption or carbon dioxide production was being measured. The oxygen consumption and the carbon dioxide production were measured on the same insect with the same manometer by first determining the manometer change resulting from the simultaneous oxygen uptake and carbon dioxide production. From this combined manometer change the carbon dioxide production rate was determined algebraically by adding the oxygen consumption rate determined in a succeeding equivalent period of time. Through a series of preliminary determinations it was found that the respiratory exchange was the same in both periods; thus, the values obtained could be used in calculating the respiratory quotient.

The average oxygen consumption and carbon dioxide production per gram of insect tissue were determined by the formulas

$$\text{Oxygen consumed} = \frac{h \cdot kO_2}{wt} \cdot 1,000,$$

$$\text{Carbon dioxide produced} = \frac{h \cdot kCO_2}{wt} \cdot 1,000,$$

where the oxygen or carbon dioxide is expressed as cubic millimeters per gram; h is the manometer reading for 1 hour corrected for thermobarometric changes, and in the case of the carbon dioxide representing the difference between the oxygen consumption and the carbon dioxide production; kO_2 and kCO_2 are the manometer constants for the respective gases; wt is the live weight of the insect in milligrams; and 1,000 is the number of milligrams per gram. The reader is referred to Dixon (1934) for further details as to procedure.

The values obtained for normal carbon dioxide production should be correct as determined by the Barcroft-Warburg procedure; it was assumed that the body fluids of the larvae and pupae of the honeybee are saturated with respect to carbon dioxide for the existing physiological conditions. Needham (1932, 1933) has criticized the use of the direct method for the determination of respiratory quotients, because of the possibility that some of the carbon dioxide produced during metabolism may be retained by tissues. Boell (1935), working with eggs of *Melanoplus differentialis* (Thos.), found a small amount of bound carbon dioxide during embryonic development. Attempts were made to determine the bound carbon dioxide in both larval and pupal honeybees by adding 3N hydrochloric or sulphuric acid to the intact organism in the respiration flask, but the integument of the insects was apparently impermeable to the acids. No measurable quantity of bound carbon dioxide was obtained from tissue brei of larval or pupal honeybees. The manometers were shaken at the rate of approximately one hundred complete oscillations per minute during these determinations. Bishop (1923) reported a pH value of 6.8 for honeybee larva blood and suggested that the increased hydrogen-ion concentration during metamorphosis favors histolysis. Taylor, Birnie, Mitchell, and Solinger (1934), as well as other investigators, have found the body fluids of insects to be acid. If any bound carbon dioxide existed, it would probably be in the form of carbonic acid. The results of these determinations are presented in Table 2.

The maximum oxygen consumption and carbon dioxide production for both castes occur during early larval life at the time of rapid growth. This period is characterized by the rapid synthesis of the structural proteins, lipogenesis, and a storage of carbohydrate reserves. With the onset of pupation the rate of metabolism is reduced to a minimum, to be followed by a rise prior to emergence which nearly equals that in the early stage. To facilitate a comparison between the respiratory metabolism of the two castes, the data in Table 2 are presented graphically in Figures 1 and 2.

The reader's attention is called to the fact that at a given time the queens are more advanced with respect to morphological differentiation than the workers; for example, the queens pupate between the fifth and sixth days, whereas the workers do not transform until approximately the eighth day. There is a similarity in the curves, although the rate of metabolism is higher for the queens throughout the larval and pupal stages. The

marked reduction in respiratory rate per gram of tissue occurs during the period of the most rapid increase in body weight. Observations made on various vertebrates and invertebrates indicate that the growth-rate decreases from birth to maturity and that the greatest decrease is found early in life. In the early stages of animal development the

TABLE 2
AVERAGE OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION OF QUEEN AND WORKER HONEYBEES PER UNIT WEIGHT OF TISSUE PER HOUR

AGE (DAYS)	NUMBER OF INSECTS	OXYGEN CONSUMED		CARBON DIOXIDE PRODUCED	
		Mean Volume (Cubic Milli- meters per Gram per Hour)	Coefficient of Variation (Per Cent)	Mean Volume (Cubic Milli- meters per Gram per Hour)	Coefficient of Variation (Per Cent)
		Queens			
2-3.....	21	4,554 ± 166	16	5,208 ± 244	21
3-4.....	20	3,423 ± 83	10	4,130 ± 138	14
4-5.....	20	2,446 ± 68	12	3,150 ± 104	14
5-6.....	19	1,391 ± 83	26	1,336 ± 106	34
6-7.....	21	760 ± 13	8	671 ± 11	7
7-8.....	27	721 ± 7	5	634 ± 9	7
8-9.....	22	718 ± 11	7	617 ± 11	8
9-10.....	23	873 ± 28	15	728 ± 28	18
10-11.....	23	1,162 ± 16	6	965 ± 12	6
11-12.....	23	1,404 ± 34	11	1,214 ± 31	12
		Workers			
2-3.....	58	2,901 ± 74	19	4,121 ± 94	17
3-4.....	55	2,238 ± 43	14	2,746 ± 58	15
4-5.....	59	1,063 ± 22	15	1,308 ± 36	21
5-6.....	57	627 ± 20	24	710 ± 22	24
6-7.....	56	409 ± 6	12	425 ± 6	11
7-8.....	57	374 ± 4	9	384 ± 5	11
8-9.....	56	396 ± 11	21	417 ± 10	19
9-10.....	64	414 ± 3	5	401 ± 4	8
10-11.....	57	415 ± 3	6	399 ± 4	9
11-12.....	58	466 ± 5	8	445 ± 5	9
12-13.....	57	509 ± 4	7	482 ± 5	8
13-14.....	59	629 ± 6	8	593 ± 8	10
14-15.....	58	754 ± 6	6	692 ± 7	8
15-16.....	56	818 ± 12	11	759 ± 13	13
16-17.....	65	1,025 ± 16	13	963 ± 18	15

chemical reactions are such as to bring about the synthesis of compounds which are to serve for structural and reserve purposes, such as proteins, fats, and carbohydrates.

The rates of oxygen consumption and carbon dioxide production per individual insect were obtained by multiplying the average weight, as given in Table 1, by the average rates of oxygen consumption and carbon dioxide production for the same age, as given

in Table 2. These figures have been plotted in Figures 3 and 4. The similarity in the development of the queens and workers and the more rapid rate of respiratory exchange of the reproductive caste shown in Figures 1 and 2 are again evident. However, when

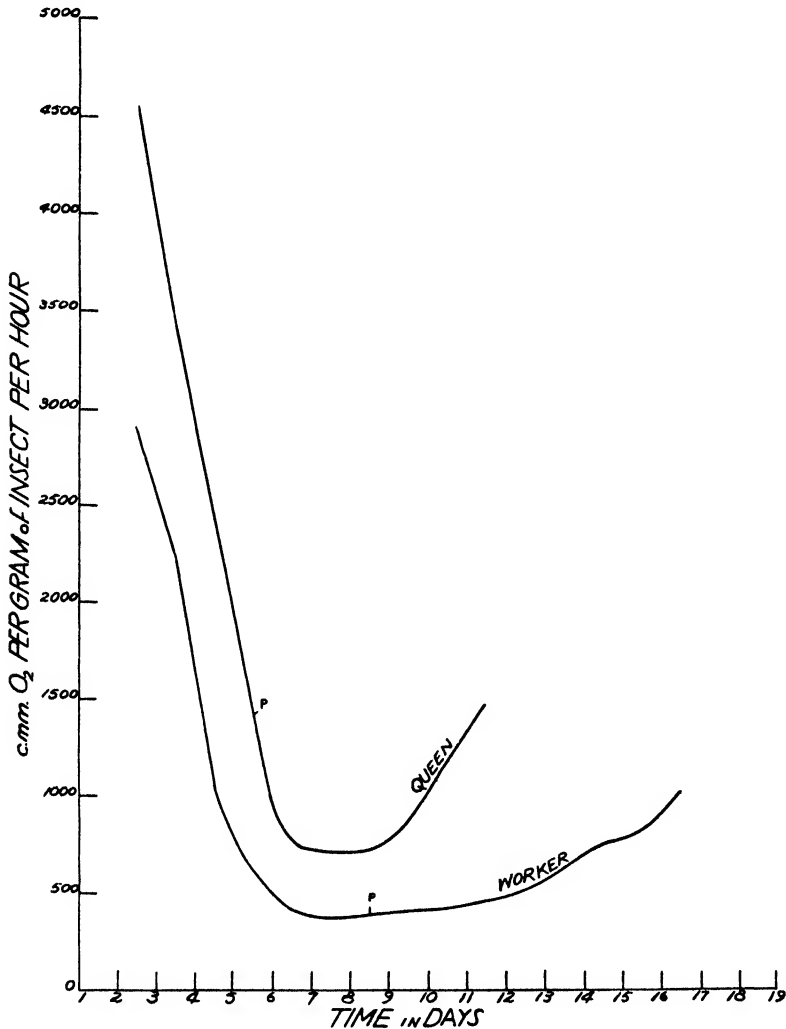


FIG. 1.—Oxygen consumption per gram of insect tissue per hour during larval and pupal development of the queen and worker honeybees. *P*, time of pupation.

calculated on an equivalent basis (per gram of tissue per hour), the rate of respiratory metabolism decreased during the larval period, whereas it showed an increase when calculated on the basis of an individual insect because of the increase in mass. The increase in respiratory rate prior to emergence is related to the development of the imaginal disks which are formed early in larval life and wait until the pupal stage before they start growing.

The pronounced physiological effect of the change in the diet on the worker is clearly demonstrated. It is of considerable interest that the queen pupated at an oxygen consumption of 355 cu. mm. per insect per hour, whereas the worker transformed at an oxy-

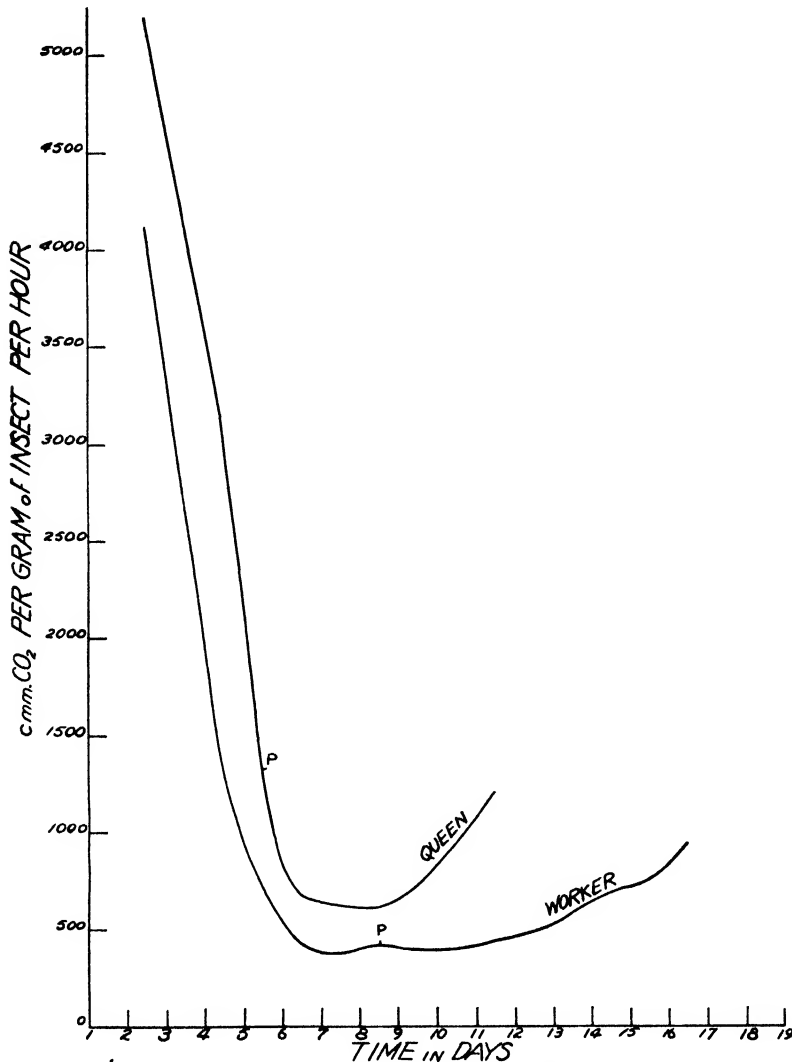


FIG. 2.—Carbon dioxide production per gram of insect tissue per hour during larval and pupal development of the queen and worker honeybees. *P*, time of pupation.

gen consumption of 50 cu. mm. per insect per hour. The queen weighed, on an average, 256 mg. at the time of pupation; and the worker, 134 mg.

It is evident that differentiation between the two castes is well under way by the second day of larval life. In the experimental procedure used, the newly hatched larvae

(less than 12 hours old) were placed in queen cells in queenless colonies, and the larvae were supplied with more food than larvae of the corresponding age in worker cells; hence the accelerated development. Figures 3 and 4 demonstrate that physiologically the members of the worker caste begin postembryonic development with the capacity to grow

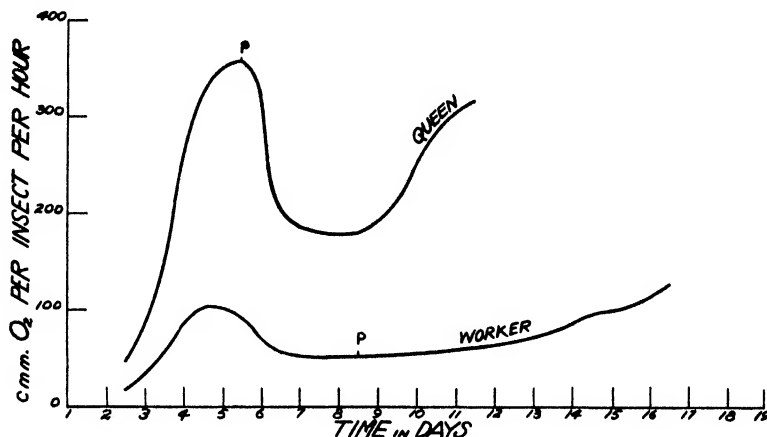


FIG. 3.—Oxygen consumption during larval and pupal development per individual queen and worker honeybee. P, time of pupation.

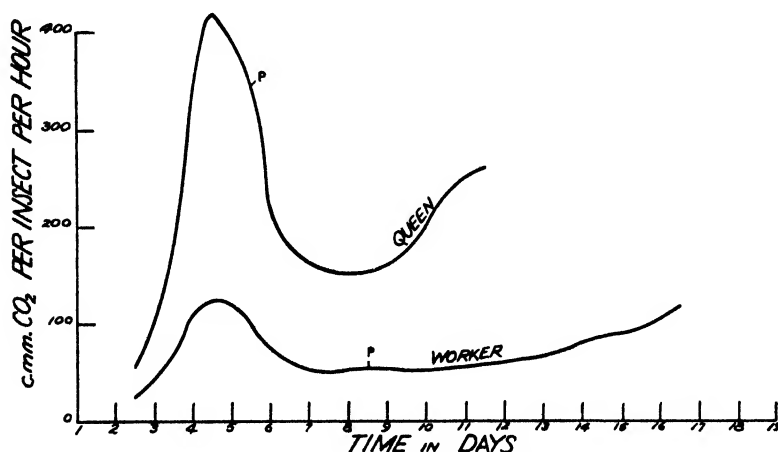


FIG. 4.—Carbon dioxide production during larval and pupal development per individual queen and worker honeybee. P, time of pupation.

similar to that of larvae which develop into the queen caste. Figures 3 and 4 indicate further that the growth per individual is accompanied by an increased respiratory exchange during larval and late pupal development. Zander and Becker (1925) found, in their work on the influence of larval age upon the development of the organs of the queen, that the critical age was between 78 and 90 hours. Queens developing from these individuals were of a transition type. Larvae over 90 hours old produced only workers.

Several workers have investigated the respiratory metabolism of other species of insects during the pupal stage and have found a typical U-shaped curve, indicating that oxygen consumption and carbon dioxide production are high at the beginning, decrease, and then rise to a maximum. This general finding is in agreement with the results presented in Figures 3 and 4. The curves for the worker caste do show a decline in the rate of metabolism following the larval period and a rise before emergence, but the change in rate is not as great as in the queen curves. The reader is referred to Fink (1925), Taylor and Steinbach (1931), Bodine and Evans (1932), and Poulson (1935) for summaries of the earlier work in this field.

RESPIRATORY QUOTIENTS

The respiratory quotients calculated from the data in Table 2 are presented in Table 3 and Figure 5.

TABLE 3
RESPIRATORY QUOTIENTS DURING LARVAL AND PUPAL DEVELOPMENT
OF THE QUEEN AND WORKER HONEYBEES

AGE (DAYS)	QUEENS		WORKERS	
	No. of Insects	R.Q.	No. of Insects	R.Q.
2-3.....	21	1.14	58	1.42
3-4.....	20	1.21	55	1.23
4-5.....	20	1.29	59	1.23
5-6.....	19	0.96	57	1.13
6-7.....	21	0.88	56	1.04
7-8.....	27	0.88	57	1.03
8-9.....	22	0.86	56	1.05
9-10.....	23	0.83	64	0.97
10-11.....	23	0.83	57	0.96
11-12.....	23	0.83	58	0.95
12-13.....			57	0.95
13-14.....			59	0.94
14-15.....			58	0.92
15-16.....			56	0.93
16-17.....			65	0.94

The respiratory quotient is of physiological significance as an index to the quality and quantity of the substances oxidized during metabolism. For example, a respiratory quotient of 1.0 indicates the oxidation of carbohydrate, since one molecule of carbon dioxide is produced for each molecule of oxygen consumed. The respiratory quotient for fat is 0.70, as lipids are relatively poor in oxygen, and in the catabolism of these substances by the animal organism some of the oxygen consumed appears as water. The volume of carbon dioxide produced by the oxidation of fat is smaller than the volume of oxygen consumed; thus the ratio is less than 1. The average respiratory quotient for protein is 0.80. Low quotients may be produced by the conversion of protein and fat into carbohydrate or by the incomplete oxidation of metabolites, whereas high respiratory quotients (greater than unity) indicate the formation of fat from carbohydrate. According to Krogh (1916), the usual respiratory quotients for animals lie between 0.72 and 0.97.

However, chemical reactions yielding high or low quotients may proceed to a limited degree, and the respiratory quotient remain within its usual range.

The respiratory quotient is greater than 1 during the larval stages for both castes of the honeybee, indicating a synthesis of lipid from carbohydrates which had been previously demonstrated by chemical analysis (unpublished). The increased production of carbon dioxide during this period is due to the synthesis of oxygen-poor fats from carbohydrates which contain relatively more oxygen. The oxygen liberated during the fat synthesis is available for oxidation and will replace the same amount of absorbed oxygen in catabolic processes, and consequently the respiratory quotient will rise.

Wierzechowski and Ling (1925) reported respiratory quotients of 1.4 and 1.58 for fattening pigs that had been fed starch. Bleibtreu (1901) observed quotients up to 1.38

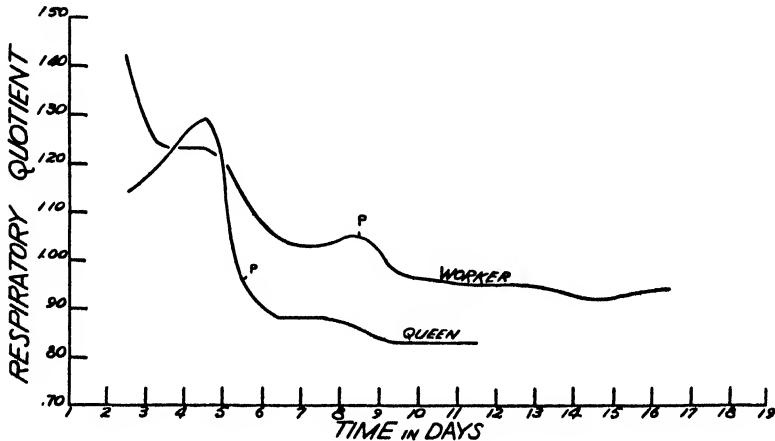


FIG. 5.—Respiratory quotients of queen and worker honeybees during larval and pupal development. P, time of pupation.

for fattening geese. Krogh (1916) has suggested that a considerable amount of carbon dioxide is produced by fermentation in the gut of certain species of animals, thus producing an increase in the respiratory quotient that is not an index of the metabolic processes. McCleskey and Melampy (1938) found that royal jelly of the honeybee has bactericidal properties. This may or may not affect bacterial flora in the gut of this insect, which in turn might influence the production of carbon dioxide. Cook (1932) reported the respiratory quotient of both normal and defaunated termites (*Termopsis nevadensis*) as approximately unity, but in starved individuals the respiratory quotient was low.

SUMMARY

Measurements of oxygen consumption and carbon dioxide production have been made on individual members of the queen and worker castes of the honeybee (*A. mellifica* L.) throughout larval and pupal development. The respiratory rates rise per individual insect during the larval period, fall to a minimum during pupation, and finally rise before emergence.

The queen or reproductive caste is characterized by a higher metabolic rate, which is associated with the more rapid growth-rate of this caste.

Respiratory quotients greater than unity indicate a synthesis of lipid from carbohydrate for both castes during the larval stages.

The respiratory quotient for the queen ranges between 0.96 and 0.83 during the pupal stage, whereas that for the worker ranges between 1.05 and 0.94. The average respiratory quotient for the queen during the pupal stage is 0.87, whereas that for the worker is 0.96.

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A DETERMINATION OF THE TEMPERATURE COEFFICIENTS FOR OXYGEN UPTAKE OF NORMAL AND BLOCKED EMBRYOS AND EGGS (ORTHOPTERA)¹

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WHILE the effects of temperature on biological systems have been investigated by many workers (see monograph by Belehradek, 1935), our knowledge of the effects of temperature on respiratory mechanisms is very vague. Since a great deal of physiological data is available concerning diapause and developing grasshopper eggs and embryos (Bodine, 1925, 1929, 1932, 1934, and Bodine *et al.*, 1934-38), it was thought desirable to investigate the temperature relations involved in the rate of oxygen consumption and to compare the responses of eggs with those of their contained embryos.

Rubenstein and Gerard (1934) have found that the Q_{10} for oxygen consumption of the unfertilized *Arbacia* egg is about twice that of the fertilized egg, and Korr (1937) has experimentally altered the respiratory rates and mechanisms of fertilized and unfertilized eggs of the same species and measured the rates at different temperatures. In view of certain similarities in the respiratory mechanisms of *Arbacia* and grasshopper eggs, a comparison of their temperature relations has been undertaken.

MATERIALS AND METHODS

Eggs of the grasshopper, *Melanoplus differentialis*, having a known temperature history and of known chronological and morphological ages, were used throughout the experiments. For the study of prediapause and diapause, eggs were placed on moist sand in a constant-temperature chamber at 25° C. (in the pods in which they were laid and not more than 24 hours after laying) until the proper age had been attained. In this paper, age of eggs or embryos is expressed as days at 25°, since Slifer (1932) has described the embryos of this species in those terms. Two thousand to eight thousand eggs or embryos constituted a series of experiments, and about twelve thousand eggs or embryos were used in all.

Diapause, a period of quiescence which eggs of this species enter at about the end of the third week of development at 25°, can be broken by subjecting them to constant low temperature for 2 months or more (Bodine, 1925, 1929). Consequently, eggs for use in the study of postdiapause were allowed to develop at 25° until well into diapause and then removed to a temperature of 5° for a period of time sufficient to break diapause. When again placed at 25°, they were allowed to develop to the 5-day postdiapause stage, by which time blastokinesis had been completed and yolk engulfment begun (Slifer, 1932). Eggs for the study of diapause were kept at 25° until they were at least 50 days in diapause.

When the eggs had reached the desired chronological age, they were removed from the pods, washed free of debris in distilled water, sterilized in 70 per cent alcohol for 10 minutes, and washed again several times in sterile distilled water. Embryos were dis-

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sected out in sterile Belar (modified Ringer) solution buffered to pH 6.3 with M/15 phosphate, and washed free of adhering yolk cells. The embryos were examined carefully, and only those of the correct morphological age were used.

Oxygen consumption was measured in microdifferential manometers, and different numbers of eggs or embryos were run in each manometer, depending on the age: 25–35 prediapause and diapause eggs or embryos were run in each manometer, and 8–10 postdiapause embryos were used. One cubic centimeter of sterile buffered Belar solution, including embryos, was used in each manometer; and the carbon dioxide produced in their respiration was removed by a small roll of filter paper soaked with 0.1 cc. of 10 per cent KOH placed in a small glass cup suspended in the reaction chamber of the manometer. When eggs were run, they were added to 1.0 cc. of sterile Belar solution.

The manometers were shaken at a rate of 75–80 oscillations per minute, which was found to be fast enough to permit adequate gas exchange without injuring the embryos. Three water baths were used, which were held constant to within $\pm 0.2^\circ$ at 15° , 25° , and 35° , respectively. Readings were taken at 15- or 20-minute intervals over a period of 1–2 hours at each temperature, and 15 or 20 minutes were allowed in each case for temperature equilibration with the manometer taps open. Except for those in the first series of experiments, eggs or embryos were run consecutively at 15° , 25° , and 35° . At the end of each experiment the embryos were again examined, and the pH of the medium was checked by colorimetric estimation with bromothymol blue.

Three temperature coefficients were calculated: the thermal increment, or temperature characteristic, μ ; Belehradek's temperature coefficient, b ; and the Q_{10} . The Q_{10} in each case was calculated from the values for oxygen consumption found in each experiment, and each value listed for Q_{10} is the mean for several experiments. The thermal increment and b were calculated from the mean values of the oxygen consumption (as mm.³ O₂/egg or embryo/hour) at 15° , 25° , and 35° . The values of μ were calculated from the familiar van't Hoff-Arrhenius equation

$$\mu = \frac{4.6 (\log K_2 - \log K_1)}{\frac{1}{T_1} - \frac{1}{T_2}}, \quad (1)$$

where K_1 and K_2 are the velocities at temperatures T_1 and T_2 , respectively, the temperature being taken in degrees of the absolute scale. The Q_{10} rule, though originally derived from a formula of Berthelot (1862), may be stated as a simplification, for small temperature intervals, of the van't Hoff-Arrhenius law. Values for this coefficient were calculated from the equation

$$Q_{10} = \left(\frac{K_2}{K_1} \right)^{10/(t_2 - t_1)}. \quad (2)$$

Belehradek (1931, 1935) has proposed an empirical temperature equation where the temperature coefficient b is calculated from the equation

$$b = \frac{\log v_2 - \log v_1}{\log t_2 - \log t_1}, \quad (3)$$

v_1 and v_2 being velocities at temperatures t_1 and t_2 , respectively.

RESULTS

Three series of experiments were carried out, the results of which are summarized in Tables 1 and 2. Table 1 lists the values found in the three series for all coefficients. In Series I the oxygen consumption of diapause and postdiapause embryos was measured, using the same temperature interval but a different sequence of temperatures (i.e., from 25° to 35° or from 35° to 25°). Since the values of all coefficients, except b for diapause embryos, vary when a different temperature sequence was used, in all the following experiments runs were started at the lower temperature in order that coefficient values might be comparable.

In the second series, diapause, postdiapause, and 15- and 18-day prediapause embryos were used. As is found with most processes, the temperature coefficients decrease

TABLE 1
TEMPERATURE COEFFICIENTS OF EGGS AND EMBRYOS

	15°-25° C.				25°-35° C.				35°-25° C.			
	No. of Expts.	Q_{10}	μ	b	No. of Expts.	Q_{10}	μ	b	No. of Expts.	Q_{10}	μ	b
Series I:												
Diapause.....					15	1.71	6,954	1.21	9	1.08	11,107	1.10
Postdiapause.....					18	1.09	1,570	0.26	4	1.28	4,314	0.70
Series II:												
Diapause.....	17	1.87	9,221	1.06	17	1.79	9,606	1.56				
Postdiapause.....	15	2.33	14,178	1.63	15	1.08	908	1.47				
Prediapause:												
15 days.....	10	2.52	15,663	1.78	10	1.65	10,207	1.66				
18 days.....	9	2.93	18,349	2.10	6	1.67	6,956	1.13				
Series III:												
Prediapause												
19-day egg.....	4	1.82	9,598	1.10	4	1.30	4,838	0.79				
19-day embryo....	4	2.50	14,705	1.69	4	1.69	9,513	1.54				
Diapause:												
Eggs.....	22	1.82	9,364	1.07	23	1.47	5,034	0.82				
Embryos.....	10	2.12	12,768	1.46	18	1.94	12,168	1.30				

with increasing temperature, although Korr (1937) found that with *Arbacia* eggs the Q_{10} increases with increasing temperature. In diapause embryos the values of μ and Q_{10} remain at about the same level, while b increases at the higher temperature range. Since the diapause embryo shows lower temperature coefficients than the developing one, it is apparently less sensitive to these temperature changes, and it might be expected that its temperature coefficients would be more constant at different temperatures. It is interesting that at 15°-25° the highest values for temperature effects on prediapause embryos occur at 18 days, when the oxygen consumption at 25° is greatest (Bodine and Boell, 1936c); whereas at 19 days, when oxygen consumption begins to decline, just before the beginning of diapause, the temperature coefficients also are lower. Explanation cannot readily

be given for the very low temperature coefficients of postdiapause embryos at the higher temperature range, both in those which were run only at 25° and 35° and those run at 15°, 25°, and 35°. Temperatures as high as 35° certainly do not have injurious effects, since Bodine (1925) has found that hatching-time is decreased when eggs are kept at this temperature.

To compare the temperature coefficients of eggs and embryos, a third series of experiments was performed, using prediapause and diapause eggs and embryos. Eggs and embryos were run on consecutive days, and embryos were always used in the same manometers which were employed for the eggs from which they were dissected. In order that

TABLE 2

MEAN VALUES AND PROBABLE ERROR OF MEAN FOR Q_{10} OF EGGS AND EMBRYOS

	15°-25° C.	25°-35° C	35°-25° C
Diapause embryos:			
Series I		1 71 ± 0 16	1 98 ± 0 12
Series II	1 87 ± 0 08	1 79 ± 0 07	
Series III	2 12 ± 0 11	1 94 ± 0 08	
Mean	1 99 ± 0 07	1 82 ± 0 06	
Prediapause embryos:			
Series II:			
15 days	2 52 ± 0 13	1 62 ± 0 07	
18 days	2 93 ± 0 17	1 67 ± 0 13	
Series III:			
19 days	2 50 ± 0 27	1 69 ± 0 03	
Mean	2 68 ± 0 10	1 67 ± 0 05	
Postdiapause embryos:			
Series I		1 09 ± 0 05	1 28 ± 0 06
Series II	2 33 ± 0 08	1 08 ± 0 05	
Mean		1 08 ± 0 04	
Diapause eggs:			
Series III	1 82 ± 0 07	1 47 ± 0 02	
Prediapause eggs:			
Series III	1 82 ± 0 24	1 30 ± 0 04	

eggs and embryos might be run at as nearly the same stage of development as possible, the eggs, after each run, were completely immersed in Belar solution and kept at 25° until the next day, when the embryos were dissected out, since Boell (1935) and Schipper (1938) have found that the development of eggs immersed in unaerated solutions is retarded. It is seen that for a given lot of diapause or prediapause eggs the temperature coefficients of the embryos are larger than those of the eggs from which they were removed.

The mean values and probable error of the mean for the Q_{10} of eggs and embryos are listed in Table 2.

DISCUSSION

It has been shown (evidence reviewed by Korr, 1937) that the respiration of the fertilized *Arbacia* egg proceeds largely through the cyanide-sensitive cytochrome-indophenol oxidase (Warburg-Keilin) system, while that of unfertilized eggs proceeds mainly through cyanide-stable nonferrous carriers. Korr (1937) has reviewed evidence and presented additional data showing that the maximum rate of substrate activation of which the dehydrogenases are capable is the same in fertilized and unfertilized eggs, and hence that the rate-controlling link in the oxidative chain is the hydrogen transfer mechanism. Rubenstein and Gerard (1934) and Korr (1937) find that the temperature coefficient for oxygen uptake of unfertilized eggs is about twice as large as that of fertilized eggs. Korr concludes, then, that "the less closely the rate of hydrogen transfer approaches the rate of substrate activation the larger the temperature coefficient; the more nearly this value is reached, the smaller the coefficient—down to the value found for the fertilized egg."

Bodine and collaborators (1934-38) have studied the effect of cyanide, methylene blue, and carbon monoxide on developing and diapause grasshopper eggs and find that with respect to respiration the developing egg behaves similarly to the fertilized *Arbacia* egg and that the responses of the diapause egg are very much like those of the unfertilized *Arbacia* egg. Their results would seem to suggest that certain of the respiratory mechanisms involved are very much alike in both cases.

At the higher temperature range (25° - 35°) the present data are somewhat variable. As shown by Table 1, diapause embryos that are run from 15° to 25° to 35° show coefficients that are about the same as those of 15-day embryos run in the same way. However, 18-day embryos have coefficients which are somewhat smaller than those of embryos in diapause. Diapause embryos run only at 25° - 35° show higher Q_{10} and μ values than any of the foregoing, while diapause embryos of the third series show Q_{10} and μ values which are still higher. Nineteen-day embryos of this series show values for all three coefficients which are practically identical with those of diapause embryos in the second series. In view of the variability of the temperature coefficients at this temperature range, certainly they cannot be used as the basis for any definite statements about the respiratory processes involved.

However, in the lower temperature range (15° - 25°) the data are more consistent. As shown by Table 2 the temperature coefficients of diapause embryos are considerably smaller than those of either prediapause or postdiapause developing ones. While the respiratory processes of fertilized and unfertilized *Arbacia* eggs do show certain similarities to those of developing and diapause grasshopper eggs, respectively, the fact that fertilized *Arbacia* eggs have lower temperature coefficients than unfertilized ones makes it evident that some aspects of their respiration cannot be the same. It is impossible to determine from these experiments just what the differences may be. The Q_{10} of diapause eggs is about the same as that found by Bodine and Thompson (1935) on diapause eggs used as controls, making allowance for the somewhat different temperature interval (20° - 30°) which they employed. Rogers (1929), using that temperature range, obtained a Q_{10} of 2.40 for oxygen consumption of *M. differentialis* nymphs at the second to fourth instar.

Bodine and Boell (1936c) have pointed out the necessity for considering problems of metabolic activity during embryonic development not only in terms of the egg as a whole but also in terms of the contained embryo. It is apparent from the data in Table 1 that

this point of view is justified. They have found that the respiratory rate of embryos is lower than that of whole eggs throughout the entire period of embryonic development. Since the respiration of the yolk cells and embryonic cells may not necessarily be governed by exactly the same processes (and it would seem that they are not), it is not surprising that the temperature responses of embryos and whole eggs are somewhat different. The coefficients obtained using the intact egg must be resultant values of the accelerative effect of increased temperature on the yolk cells and extra-embryonic membranes plus the embryonic cells themselves. Although stimulation cannot definitely be ruled out as a possible factor in the higher-temperature coefficients of the embryo, since the resultant values are less both in diapause and prediapause (Table 1) than those for the embryo, it would appear that the yolk cells are not affected by temperature to as great an extent as is the embryo. It is interesting that in prediapause the yolk cells and serosa cells are very resistant to X-rays while the embryo is quite sensitive to them (unpublished data of Dr. E. E. Carothers; see Ray, 1938).

SUMMARY

1. Temperature coefficients for oxygen consumption of *M. differentialis* eggs and embryos of different ages and in different physiological states have been determined at two temperature intervals (15° – 25° and 25° – 35°).
2. Different values for the coefficients are found when different sequences of experimental temperatures are employed.
3. In the lower temperature range the coefficients of diapause embryos are lower than those of either prediapause or postdiapause developing ones. In the higher temperature range no significant differences between responses of diapause and prediapause developing embryos can be seen. Postdiapause embryos are practically insensitive to temperature changes from 25° to 35° .
4. Diapause and prediapause embryos have higher coefficients than the eggs from which they were removed. The probable significance of this is pointed out.

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RETARDATION OF EARLY CLEAVAGE OF URECHIS BY ULTRA-VIOLET LIGHT

(One plate and one figure)

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ULTRA-VIOLET light is known to have strong effects upon protoplasm and therefore has served as a useful tool in biological investigations. Yet we know comparatively little as to the mode of action of these radiations and so cannot use them most effectively. Quantitative studies of a comparative nature should throw some light on this complex problem. In a preceding study (Giese, 1938a and 1938b) effects of known dosages of monochromatic ultra-violet light upon the cleavage of sea-urchin eggs were reported. In the following paper a comparable study on the effects of ultra-violet light on the cleavage of the marine worm *Urechis caupo* Fisher and MacGinitie is presented.

The animals used in this study were collected during the spring and summer of 1938 at Bolinas Bay, California. They were transported to the laboratory in pre-cooled food jugs and kept two or three per gallon jar in 500-1,000 cc. aerated sea water (obtained at Bolinas Bay) in a constant-temperature room ($15 \pm 0.5^\circ \text{C.}$). The sea water was changed whenever necessary. Under such conditions the worms remained healthy in many cases for as long as a month.

Eggs and sperm were drawn from the segmental organs with fine pipettes, one organ being entered at a time and each organ only once, a chart being kept for the removal of materials from each animal. In experiments with monochromatic light the eggs and sperm were irradiated at room temperature ($20^\circ\text{--}23^\circ \text{C.}$) but were quickly returned to the constant-temperature room, where all observations were made. The equipment and procedures were similar to those previously used (Giese, 1938a and 1938b). Observations were made at intervals of 15-45 minutes to determine when most of the eggs had undergone a cleavage.

One drop of a sperm suspension containing about 1 part of the spermatid fluid to 200 parts of sea water was used for each 250-300 eggs contained in about 1 cc. of sea water. Even when all the eggs showed fertilization membranes after insemination, a few failed to cleave and some underwent abnormal cleavage, but the others showed normal and rhythmic cleavage and gave rise to apparently normal trochophores. Tyler (1931) has described the early development of *Urechis*; the reader is referred to his paper for details.

EXPERIMENTAL

In the first series of experiments the eggs and sperm were irradiated with a graded series of exposures 41 mm. from the center of a mercury-argon discharge tube, which emits radiations about 88 per cent of which (excepting the heat rays) are of $\lambda 2537 \text{ \AA}$ (Leighton and Leighton, 1935), to explore more rapidly the various types of effects resulting from the irradiation with ultra-violet light. The more interesting effects were then investigated, using light of two wave-lengths obtained from a monochromator, viz.,

λ 2654 A and λ 2804 A. These two lines were chosen because the first lies near the region of maximal absorption by nucleoproteins (Heyroth and Loofbourow, 1933) and the second near a maximum for cytoplasmic proteins (Coulter, Stone, and Kabat, 1935).

TABLE 1
EFFECTS OF RADIATIONS OF THE MERCURY-ARGON DISCHARGE TUBE
UPON EGGS AND SPERM OF *Urechis*

Dosage in Seconds	Sperm Irradiated, Then Used To Inseminate Normal Eggs	Eggs Irradiated Not Fertilized	Eggs Irradiated, Then Fer- tilized with Normal Sperm
0	Normal fertilization Normal cleavage	No change observable	
4	Normal fertilization mem- branes Early cleavage almost nor- mal, later somewhat ab- normal	No change observable	Normal fertilization mem- branes Retarded and somewhat ir- regular cleavage Abnormal gastrulae
8	Normal fertilization mem- branes Early cleavage almost nor- mal, later abnormal	No change observable	Normal fertilization mem- branes Retarded and irregular cleav- age Small percentage motile lar- vae
16	Normal fertilization mem- branes Some inactivated Abnormal cleavage	Activation of some Membranes appear normal Cleavage of many of those activated Abnormal motile embryos	Normal fertilization mem- branes Abnormal cleavage Occasional motile larvae
32	Most inactivated (all in two trials) Abnormal and occasional cleavage (no cleavage in two trials)	Activation of most Cleavage of small propor- tion, abnormal	One-sided crinkled fertiliza- tion membranes Nuclei divide but only oc- casional abnormal cleav- age
64	Inactivated No fertilization membranes induced	Activation; abnormal mem- branes on almost all Some became multinucleate Cleavage rare and abortive	One-sided crinkled fertiliza- tion membranes Nuclei divide but cleavage rare
128	Inactivated No fertilization membranes induced	Activation; abnormal mem- branes on most Germinal vesicle may break down but no cleavage oc- curs Early cytolysis	Abnormal fertilization mem- branes Neither nuclear nor cyto- plasmic division Early cytolysis

1. *Irregular cleavage after irradiation and its causes.*—The more interesting effects of the mercury-argon lamp upon the eggs and sperm are summarized in Table 1. As can be seen from this, irradiation produces both (1) retardation of the rate of cleavage and (2) irregularities in cleavage. The irregularities in cleavage were observed to be of three types: (a) a greater distribution of cleavage stages (e.g., while controls seldom showed more than two stages at the same time [cf. 4- and 8-celled stages], those ir-

radiated showed three or even four stages [cf. 1-, 2-, 4-, and 8-celled stages] at the same time); (b) stages not normally present except transiently or occasionally, such as the 3-celled, 5-celled, 6-celled, etc., in which cleavage of certain blastomeres lags behind that of others; (c) completely abnormal stages in which large- and small-sized, as well as irregularly shaped, blastomeres occur. Some irregular cleavages are illustrated in Plate I, Figures 5-16.¹

These irregularities in cleavage may be due to (1) polyspermy in some cases; (2) suppression of polar-body formation after larger dosages in others; and (3), probably in most cases, an effect of the radiation on the processes which regulate distribution of nuclear and cytoplasmic materials in cleavage as well. Thus, a large number of the eggs irradiated for 4 seconds gave rise to 3, 4, 5, or even more cells in the first cleavage. These eggs both showed polar bodies and underwent the first cleavage at about the same time as the controls. Since ultra-violet light does not generally seem to stimulate nuclear divisions, the division of the egg into more than 2 cells at about the time controls gave rise to 2 cells most probably indicates polyspermy. At higher dosages and in a small proportion of cases at lower dosages polar bodies were not observed to form (occasionally after a dose of 8 seconds, rarely after a dose of 16 seconds). In such cases irregularities in cleavage might be due to suppression of polar-body formation or such suppression in addition to the polyspermic condition. Chase (1937) has demonstrated that, when the dosage is great enough, the polar bodies are prevented from forming in *Urechis* eggs.

But, even when neither of these could occur, as when eggs were irradiated just after the first cleavage, irregularity in cleavage was observed to occur. While the effects appeared somewhat later in such cases, nonetheless, when the dosage was sufficient, the same types of irregularities occurred. In this case the irregularities must be due to interference with the processes which regulate the regular distribution of nuclear and cytoplasmic materials.

2. *Effect of ultra-violet light of λ 2654 Å and λ 2804 Å on Urechis eggs.*—If one were to use the 2-celled stage for irradiation experiments, it would be possible to avoid polyspermy and suppression of polar bodies; but, because of a fairly long latent period, it is impossible to get a measure of the true retardation of the earlier cleavages. Therefore, in the experiments with monochromatic light, eggs were irradiated 30 minutes after insemination. In this case it is possible to avoid polyspermy, but after large dosages polar-body formation is prevented.

Eggs were placed in an all-quartz cell 5.845 mm. in diameter and irradiated with light from the monochromator. The maximal intensity delivered by the arc was utilized at all times; but, since this intensity decreased as the experiments were performed, the intensity varied. For λ 2654 Å the intensity varied from 2.83 to 4.25 ergs/sec/mm²; for λ 2804 Å, from 3.36 to 4.95 ergs/sec/mm². The range is relatively small in each case, and the order of magnitude for the two wave-lengths comparable.

Eggs given dosages of about 70 ergs/mm² at λ 2804 Å and about 65-270 ergs/mm² at λ 2654 Å seemed to cleave comparably to controls; but when the dosage was increased, eggs showed retardation, and with sufficient dosage, irregularity in cleavage. Dosages up to 1,000 ergs/mm² were mainly retarding (irregularities rare at smaller dosages but appearing more frequently with increase in dosage); between 1,000 and 2,000 ergs/mm², produce irregular cleavage in many; between 2,000 and 3,000 ergs/mm², produce ir-

¹ The author is indebted to Mr. E. A. Reed for making all the photographs used in this paper.

regularities in most. Following dosages between 3,000 and 4,000 ergs/mm², only a small percentage of the eggs ever underwent cleavage, and then only abnormally, while dosages of over 4,000 ergs/mm² were followed shortly afterward by cytolysis.

The irregularities in cleavage render collecting comparative data following larger dosages very difficult. The data here presented for the larger dosages are therefore not as accurate as the data published for the sea-urchin egg.

In practically all cases data for the two wave-lengths were obtained by using eggs from the same female and sperm from the same male, since it is possible to use one animal for six experiments (draining one egg or sperm segmental organ at a time; see Fisher and MacGinitie, 1928, for the structure of *Urechis*). Seven such series of experiments were performed, using the eggs and sperm of five different males and females.

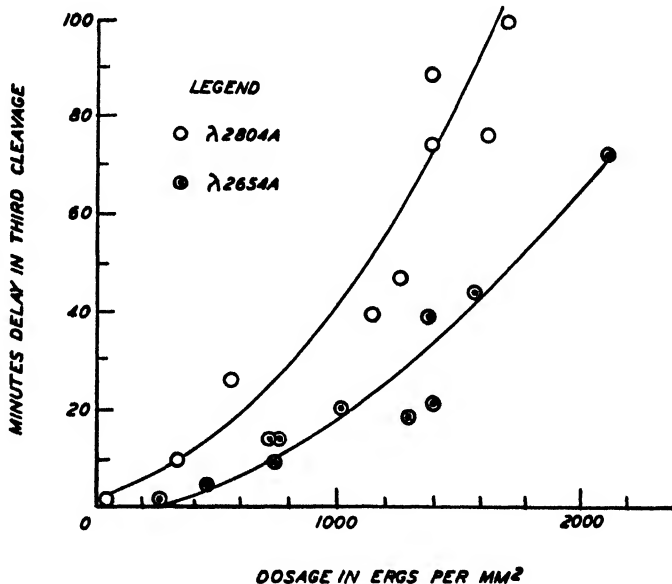


FIG. 1.—Retardation of third cleavage by irradiation with λ 2804 Å in A and λ 2654 Å in B

For comparison of the effect of the radiations the delay in the third cleavage was chosen, for reasons already discussed (Giese, 1938b). The data are plotted in text Figure 1. Study of this figure will disclose that, while there is a large spread in points, owing in part to the difficulties already noted and in part to variations in responses of eggs of different females, there is a trend indicating greater efficiency of λ 2804 Å than of λ 2654 Å in retarding cleavage of the eggs.

To gain an idea of the number of quanta required to produce retardation, the amount of light extinguished by the eggs was next determined by interposing a cell—first empty, then full of eggs—between the light source and the thermopile. By counting the eggs, the amount of radiation extinguished by a single egg could be determined. The details of the method are similar to those already described for the sea urchin (Giese, 1938b). Eggs were used in each set of extinction measurements approximately 30–50 minutes after insemination. The area of an egg extinguishing light is equivalent to the optical

sectional area. To determine this optical section, the two diameters (since some of the eggs are somewhat ellipsoidal) of 30 eggs were measured by projecting photographs of eggs taken 38 and 64 minutes after insemination; the average diameter was found to be 105.7μ in both cases. An egg of this diameter has an optical section area of $8,758.3 \mu^2$. Knowing the area of a single egg and the numbers of eggs used, one can readily determine the number of layers of eggs exposed in each of the trials. The data are given in Table 2.

TABLE 2
EXTINCTION OF ULTRA-VIOLET LIGHT BY *Urechis* EGGS

EXPT. No.	No. OF EGGS	LAYERS OF EGGS (L)	I - I*/I ₀ FOR—		COMPARATIVE EXTINCTION† AT—	
			λ 2654 Å	λ 2804 Å	λ 2654 Å	λ 2804 Å
1.....	3,854	1 258	0 885	0 912	97 0	100
2.....	2,360	0 770	0.685	0 725	94 5	100
3.....	3,558	1.166	0 830	0 864	96 0	100

* I = intensity of transmitted light; I₀, of the incident light.

† Setting extinction at λ 2804 Å = 100 in each case.

As can be seen from the table, the difference in extinction at the two wave-lengths is not very large, but it is observed in each of the experiments tried. To get an idea of the fraction of the light falling upon a single egg which is extinguished, the data for experiment No. 2 were used, and it was found that approximately 88 per cent of the incident light at λ 2654 Å and 94 per cent of the light at λ 2804 Å would be extinguished.

To obtain the quantity of energy extinguished by a given egg before a given effect is produced, it is merely necessary to multiply the incident energy in ergs per square millimeter required to produce a given effect by the area of the egg ($8,758 \mu^2$ or 0.008758 mm.^2) and by the fraction of the incident light extinguished by the egg (approximately 94 per cent at λ 2804 Å). To produce a delay in the third cleavage of about 1 hour, approximately $1,250 \text{ ergs/mm}^2$ are needed. This means that 1.46×10^{12} quanta have been extinguished. Of the quantity extinguished, only part are truly absorbed; part are reflected and scattered. But the order of magnitude of the quantity needed is not altered even if we should assume that half or even more than half were merely scattered.

3. *Effect of ultra-violet radiations on Urechis sperm.*—Hinrichs (1926) has described abnormal development of sea-urchin eggs fertilized by irradiated sperm. The sperm is almost a naked nucleus, and it seemed interesting to compare its resistance to ultra-violet light with that of the egg. The sperm suspension removed from a male *Urechis* was diluted to 1:200 with sea water in the first two series of experiments and to 1:1,000 in the last series.

The intensity of the light was comparable to the intensity used for the eggs. A dosage series from 158 to 3,020 ergs/mm² was used at λ 2654 Å, and a dosage series from 189 to 4,328 ergs/mm² was used at λ 2804 Å. Even after the largest dosages given, some sperm retained their motility and some were able to evoke membrane formation on the eggs. Longer dosages were not tried, since after about an hour sperm begin to lose potency even without irradiation at the dilutions used.

After dosages as high as 3,020 ergs/mm² for λ 2654 A and 4,328 ergs/mm² at λ 2804 A had been given the sperm, polar-body formation of eggs inseminated with the sperm and the first and second cleavages occurred in almost the normal manner and at almost the same time as in the controls. For the smallest dosages the cleavages were indistinguishable from those of the controls. But generally by the third cleavage the injurious effects of the irradiation of the sperm were quite apparent. Eggs fertilized by sperm which had been given large doses of the radiations showed at this time either (1) extremely abnormal division of at least one-half of the cells or (2) the disintegration of one-half of the cells into small globules. The effects were most pronounced and appeared earlier, the greater the dosages given. Irregular cleavage following insemination by irradiated sperm is illustrated in Figures 17-20 (Pl. I).

A few measurements of the extinction of ultra-violet light of these two wave-lengths by sperm were made and are summarized in Table 3. As can be seen from the table,

TABLE 3
EXTINCTION OF ULTRA-VIOLET LIGHT BY *Urechis* SPERM

EXPT. No.	APPROXIMATE DI- LUTION OF SPERM	1 - I/I ₀ FOR—		COMPARATIVE EXTINCTION* AT—	
		λ 2654 A	λ 2804 A	λ 2654 A	λ 2804 A
1.....	1:80	0 529	0 460	100	86 9
2.....	1:40	0 754	0 657	100	87 1
3.....	1:40	0 800	0 670	100	83 7
4.....	1:40	0 827	0 710	100	85 8
5.....	1:40	0 848	0 740	100	87 3

* Setting extinction at λ 2654 A = 100 in each case.

more light is extinguished by the sperm at λ 2654 A than at λ 2804 A, as might be expected of cells containing a large proportion of nuclear material. From this one might expect a greater action of λ 2654 A than of λ 2804 A upon the sperm. Unfortunately, from the nature of the data it is impossible to be objective, and the attempt to decide between the comparative efficiency of the two wave-lengths was abandoned. In any case, the efficiency of the two wave-lengths in producing the irregular development is of the same order of magnitude.

Ultimately the eggs fertilized with sperm given smaller dosages of radiations formed blastulae, some of which gastrulated and finally formed trochophores. Eggs fertilized with sperm given larger dosages developed abnormal swimming forms, often consisting of a large cell and, attached to one end of it, a bunch of small cells, looking, in many instances, like a balloon and a basket. Such abnormal embryos merely revolved in circles. The data are summarized and compared to the data for the eggs in Table 1.

DISCUSSION

Irradiation of *Urechis* eggs with ultra-violet light has two main effects upon cleavage of the eggs: (1) retardation of the rate of cleavage and (2) production of irregular cleavage. The retardation of cleavage is probably due to interference with reactions which

govern the rate of cleavage, while the irregularity is probably due to interference with processes which govern the regular distribution of materials. Under certain conditions irregularities may be due to polyspermy or suppressed polar-body formation as well. In the *Urechis* egg the threshold for (1) is high; thus, whereas a dosage of 1,000 ergs/mm² at λ 2804 Å will produce delay at the third cleavage of about 40 minutes in *Urechis*, such a dosage produces a delay of about 2½ hours in the sea-urchin egg. The threshold for (2) in the *Urechis* egg, however, is lower than that of the sea-urchin egg, for irregularities are readily produced even at intermediate dosages, whereas sea-urchin eggs must be given more prolonged dosages to evoke comparable irregularities.

The membrane of the *Urechis* egg is quite readily altered by irradiation. Thus, when the egg is irradiated with small dosages of radiations from the mercury-argon discharge tube, the fertilization membranes form even faster than in controls; but when the dosage is larger (over 16 seconds), the membrane becomes more crinkled than in controls and tends to be one-sided. Dented eggs (see Tyler, 1932, for a discussion of the types of eggs of *Urechis*) round out quickly after insemination; those irradiated for less than 16 seconds with the discharge tube develop dents again; those irradiated for more than this dosage do not, indicating various osmotic effects. Unfertilized eggs of *Urechis* irradiated with the mercury-argon discharge tube with appropriate dosages were found to become activated (irradiation of the medium for even twenty times as long had no effect). Some of the data are given in Table 1. This activation is in striking contrast to the situation obtaining for the sea-urchin egg, where comparable dosages produced no activation (Giese, 1938a).

The greater efficiency of λ 2804 Å over λ 2654 Å in retarding cleavage of the eggs suggests a greater effect on the cell cytoplasm than upon the nucleus, although the nucleus is undoubtedly affected as well, since nucleoproteins absorb strongly at both of these wave-lengths (Caspersson, 1936). The fact that the sperm is so highly susceptible to the radiations indicates nuclear sensitivity to both wave-lengths and suggests that the nucleus of the egg, which seems to be less affected, is probably protected at both wave-lengths by the mantle of cytoplasm which incloses it. Unfortunately, the effects on egg and sperm are qualitatively different and do not allow of direct quantitative comparison.

Following irradiation of eggs occurs a latent period before the effects are fully expressed and definitely observable, although slight retardation is early detectable. Thus, the toxic photoproducts produced by the radiations do not seem to produce a full effect until a certain time-interval has elapsed. Possibly this period is the time required for the diffusion of the toxic materials from the seat of formation. When irradiated sperm are used to inseminate normal eggs, the first two cleavages show slight retardation, the effect being expressed fully by about the third cleavage. This suggests that the units introduced by the sperm act for a time in almost a normal manner before disintegration and full expression of the effect occurs. The sperm of *Urechis* are not readily inactivated by the radiations used, but irradiated sperm produce irregularities in cleavage (generally after the second cleavage) following fairly small dosages. The sperm of the sea urchin, on the other hand, are more readily inactivated and rendered impotent, and the effect on cleavage is also more quickly expressed, than in the former case (Giese, 1939).

The number of quanta required to produce a given retarding effect in both *Urechis* and sea-urchin eggs is of the same order of magnitude, although, as already pointed

out, there are differences in susceptibility of the two eggs. The number of molecules involved is, however, in both cases very large.

The differences in response to ultra-violet radiations of the eggs and sperm of the sea urchin and of *Urechis* probably depend upon differences in their structure and physiological nature. Thus, the *Urechis* egg is larger than the sea-urchin egg (105.7 μ compared to 77.2 μ in diameter for the sea urchin). The *Urechis* egg extinguishes more of the incident light than does the sea-urchin egg (e.g., 76.3 per cent at λ 2804 Å for the sea-urchin egg, 94 per cent for *Urechis*). The egg of the sea urchin contains a yellowish pigment; the egg of *Urechis*, a granular reddish pigment. The egg of *Urechis* has a more rapid rate of cleavage and of differentiation than that of the sea urchin. The more active egg may thus repair injuries more quickly than the less active one. On the other hand, the cleavage of the *Urechis* egg is rendered irregular with smaller dosages of radiations than is the sea-urchin egg. This may indicate differential susceptibility in the two eggs of the reaction chains governing the rate of cleavage, on the one hand, and the regularity of cleavage, on the other.

The effects above enumerated, as well as other effects previously discussed, indicate that the action of the radiations on the eggs is a summation of the effect on various cell structures and functions. The vast number of quanta involved in producing the effects also suggests a complex series of reactions following the absorption of the light by the cell.

SUMMARY

1. Following irradiation with ultra-violet light, the eggs of *Urechis* were found to undergo retarded and irregular cleavage.
2. Retardation, as well as irregularity, increased with increase in dosage.
3. Irregularities were of three kinds: (1) presence of more developmental stages at one time, (2) lag in cleavage of some blastomeres, and (3) unequal and irregular divisions.
4. Retarded and, following larger dosages, irregular cleavage occurred whether the unfertilized egg, the egg 30 minutes after insemination, the 2-celled stage, or the sperm were irradiated with sufficient dosages.
5. A latent period followed the irradiation before the effects of the absorbed radiation were fully expressed.
6. Activation occurred when unfertilized eggs were given certain dosages of radiations from the mercury-argon discharge tube.
7. Polyspermy occurred when unfertilized eggs were given certain dosages and were subsequently inseminated.
8. Of the two wave-lengths, λ 2804 Å and λ 2654 Å, the first was somewhat more effective in causing retardation than the second.
9. The extinction of radiations by the *Urechis* eggs is of the same order of magnitude for each of these wave-lengths, being slightly larger for λ 2804 Å than for λ 2654 Å.
10. The extinction of radiations by sperm is greater at λ 2654 Å than at λ 2804 Å.
11. The number of quanta needed to produce retardation of cleavage of the eggs is large, being of the order of magnitude of 10^{22} to produce a delay of about 1 hour in the third cleavage after irradiation with λ 2804 Å.

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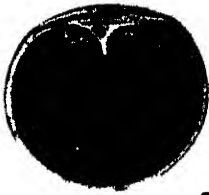
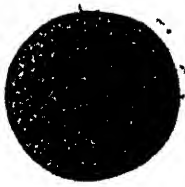
PLATE I

Figs. 1-4.—Stages in normal development.

Figs. 5-16.—Abnormalities in development of irradiated eggs.

Figs. 17-20.—Abnormalities in development of eggs fertilized by irradiated sperm.

PLATE I



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GROWTH STUDIES ON CILIATES. I. THE ROLE OF BACTERIA IN THE GROWTH AND REPRODUCTION OF COLPODA¹

(Two figures)

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THE various species of the genus *Colpoda* have been the subject of numerous and interesting investigations for many years. From the standpoint of the cell physiologist these ciliates offer unusual material in that they are relatively easy to culture, they have a high-division-rate, and they form permanent cysts. This last fact has made them the subject of a great many investigations regarding the nature and causes of encystment and excystment. Of the various attempts to analyze these problems none, we feel, have been done under conditions which would permit a proper control over extraneous factors, of which a principal one is associated bacteria. We have succeeded in controlling this factor and along with it many others that enter into the problem.

It is the purpose of this report, which is the first of a series, to detail our experiments which have been carried on for well over a year. It is hoped that we can show the necessity of, and the consequences of the lack of, a precise method for bacterial control in dealing with the culture methods for studies of ciliate metabolism in general. While the majority of the data given regarding the effects of differential feeding are of a qualitative nature, the observations here recorded are, we believe, of extreme significance.

Our strain of the ciliate *Colpoda*² came from a single individual isolated from an infusion of Florida hay in the same manner as previously described for *Colpoda cucullus* (Kidder and Claff, 1938). The progeny of this individual were grown for several months on boiled hay infusion, and careful records were made of the growth-rate, time of encystment, time and method of excystment, as well as of the cytological details during the

¹ This investigation was supported in part by a grant-in-aid from the Society of the Sigma Xi.

² Since the completion of this study we have received cultures of *Colpoda duodenaria* from Dr. C. V. Taylor, and it appears that our strain is morphologically identical with it. An analysis of the genus is now in progress in this laboratory, and until this is completed we should prefer to withhold specific designation of our strain.

various phases of the life-history. These records have been used for comparative purposes throughout the later periods of the investigation.

In January, 1938, the initial investigations into the effects of bacteria on the growth, reproduction, and cyst formation began. Because of previous experience in the feeding of bacteria to *Moina macrocopa* (Stuart *et al.*, 1931) it was decided that a likely food organism with which to start would be *Aerobacter cloacae*, a common coliform bacterium found abundantly in soil and water. This bacterium has proved to be not only adequate as food for our strain of *Colpoda* but far superior to any other type or combination of types which we have used.

It is obvious and has been mentioned many times in the literature within the past few years that in order to test the effects of any given food material on a bacteria-feeding protozoan, that protozoan must be absolutely freed of contaminating microorganisms. Our first attempt at sterilization consisted of a method of washing single trophic ciliates in ten changes of sterile medium, either dilute proteose-peptone broth, hay tea, or distilled water, transferring between each bath with sterile micropipettes. All dishes were sterilized before use and kept covered throughout the process except for the time needed to effect the transfer. This method is similar to that described by Hargitt and Fray (1917) and Parpart (1928). After the tenth transfer, the whole process consuming about 3 hours, the individual ciliates were placed in bacteriological tubes containing different media.

We were anxious to prevent, as far as possible, the growth of any contaminating microorganisms as well as those bacteria which we were presenting as food. To this end we set up tubes containing *Aerobacter* suspended in Pyrex distilled water, without the addition of any of the salts which had previously been used in studies of this kind (see Barker and Taylor, 1931). We wanted to test the theory that, even if our method of sterilization proved to be ineffective, it would, nevertheless, reduce the number of contaminating microorganisms to such a low number that they would be eliminated in successive transplants in *Aerobacter* suspensions. This method of substitution of bacterial types had been previously reported by Oehler (1920).

It was found that all the ciliates, washed as previously described and placed in nutritive media, showed macroscopic contaminations within 48-72 hours. Therefore, the method used was ineffective for complete sterility. Those ciliates placed in suspensions of *Aerobacter* in distilled water grew exceedingly rapidly and to enormous concentrations. At the end of 48 hours the fluid had become crystal clear, most of the *Aerobacter* having been eaten, and most of the ciliates had encysted in a ring about the tube at the surface. Here, then, was a situation which should yield results in the elimination of contaminants by transplantation. Accordingly, serial transplants were made into fresh *Aerobacter* suspensions by means of a bacteriological loop. A number of series were started in this manner, transplants being made every 48 hours. At the outset agar plates were streaked from these tubes, and contaminants were found but only in small numbers. These numbers indicated that if the fluid was actually non-nutritive these organisms would be speedily lost when transplanted through suspensions of billions of *Aerobacter*.

After a period of 6 weeks, stock was taken of our results. It was seen at a glance that something was changing in our serial transplants. Whereas the early tubes had given remarkable ciliate growth and the fluid had become clear, as time went on the ciliate growth decreased in direct proportion to the failure of the fluid to become clear after encystment had taken place. What was the cause of the persistence of cloudiness in the

later tubes? Were the ciliates failing to eat the *Aerobacter*? Agar plates streaked from these tubes demonstrated a point of great importance. These plates showed very few *Aerobacter* but were covered with extraneous types—the types which were brought in as contaminants in small numbers with the original washed ciliate. These contaminants, then, had actually increased in number to an enormous degree while the ciliates were ingesting *Aerobacter*. Upon what were the contaminants feeding, and why were they not eaten out at the same rate as were the *Aerobacter*? It seemed obvious that the *Colpoda* must be practicing some kind of selectivity in their feeding and that the contaminants were growing, possibly on the metabolites of the ciliates or on the disintegration products of the bacteria or both. Certain it was that the fluid had become nutritive during the period of ciliate growth. This meant, therefore, that at each transplant the contaminant inoculation would be, even in a loop, heavier and heavier. The conclusions suggested by this observation—namely, that the fluid was nutritive after the ciliates had grown in it, that the ciliates selected *Aerobacter* as food in preference to the contaminants in these tubes, and that the contaminants appeared to be detrimental to the growth and well-being of the ciliates—were subjected to experimental check.

TABLE 1
EXPERIMENT TO TEST THE ABILITY OF TWO TYPES OF
FLUID TO SUPPORT GROWTH OF *Pseudomonas*

FLUID	BACTERIAL COUNTS				
	Initial	24 Hours	48 Hours	72 Hours	96 Hours
1. Filtered ciliate culture*	45,000	4,500,000	29,600,000	23,900,000	30,000,000
2. Filtered <i>Aerobacter</i> suspension†	25,000	38,000	165,000	1,480,000	21,500,000

* Filtered (Seitz) *Colpoda-Aerobacter* culture 48 hours after growth of the ciliates had started.

† Filtered (Seitz) *Aerobacter* suspension which had stood 48 hours previous to filtering.

In order to demonstrate the nutritive quality of the fluid in which ciliates have grown, the following experiments were performed. Forty-eight-hour cultures from our *Colpoda-Aerobacter* pure series (to be described later) were drawn through a Seitz bacteriological filter, and the filtrate inoculated with *Pseudomonas* (our most prevalent contaminant). The initial inoculum was determined by plate count, and the tubes incubated at 26° C. Thereafter bacterial counts were made at 24-, 48-, 72-, and 96-hour intervals. A parallel series using a filtrate of distilled water in which *Aerobacter* alone had been suspended for 48 hours before filtration was inoculated and treated in the same manner as the first. The results of these experiments are presented in Table 1 and are graphically represented in Figure 1.

The marked increase of the bacteria in the *Colpoda-Aerobacter* filtrate might conceivably be due to nutritives derived from the ciliate metabolites, bacterial disintegration products, traces of medium brought in from the agar slants during the initial bacterization 48 hours previous to filtration, or to a combination of these factors. Ciliate metabolites are eliminated from the second set of tubes, and the growth here is much slower and the concentrations lower during the first 72 hours. By the end of 96 hours the concentrations are nearly equal. It must be remembered, however, that the presence of

Aerobacter in high concentrations for a period of 48 hours (as was the case in the second part of this experiment) never occurs in the ciliate cultures, as they are rapidly eaten out. Therefore, it is not correct to compare this experimental fluid to that of a ciliate culture. Judging solely from the figures, however, it appears that a considerable quantity of nutrient material, over and above that supplied by bacterial disintegration, is supplied by some product of ciliate activity, together with what nutrients may have been brought in with the original bacterization. We believe that the nutrients brought in from the agar slants are negligible, considering the fact that the bacteria are always removed with care with a wet loop. Distilled water inoculated with *Pseudomonas* showed a slight decrease in numbers at the above-mentioned time intervals.

We feel that the foregoing results are quantitatively conservative as compared to our first observations on the growth of the contaminants in our tubes, because it must be remembered that we were testing the nutrient qualities of the fluids with bacteria previously

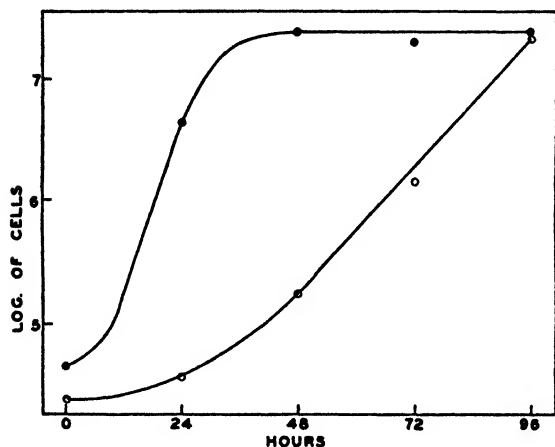


FIG. 1.—Graphic representation of the data contained in Table 1.

Aerobacter. When the ciliates had encysted, these tubes were emptied of their fluid, and the cysts, adhering to the walls, were rinsed in many successive changes of sterile distilled water using aseptic technique. This manipulation aided in removing gross contaminations. These tubes were then filled with sterile distilled water to a point where the cyst rings were just submerged. They were allowed to stand until the ciliates had excysted, and the small trophic forms were swarming in the water. Under these conditions excystment took place in about an hour, although there were some irregularities in time, the causes for which are under investigation at the present time. The advantages of this procedure were that an opportunity was offered to pre-wash the cysts and that the cytoplasm of the freshly excysted ciliate was devoid of food.

After excystment had occurred, the ciliates were concentrated by slow centrifugation, and the concentrate removed to a single, sterile, cotton-plugged centrifuge tube. This ciliate concentrate was diluted with 10 ml. of sterile distilled water and recentrifuged at a speed which would just throw down the majority of the ciliates in 3 minutes. It was found that the most satisfactory speed for this purpose was 2,000 revolutions per minute.

ly grown on agar slants. These inoculated bacteria were probably not as well adjusted to the experimental fluids as were those which had grown for 6 weeks in association with the ciliates.

Testing the other points suggested by our observations necessitated complete sterilization of the ciliates. This was effected by centrifugation and dilution. It was found, however, that slight but important modifications had to be made in the usual method in order to obtain the desired results. Our procedure, as finally perfected, consisted of growing large numbers of ciliates in tubes of distilled water bacterized with

As soon as the centrifuge stopped, the supernatant fluid (9 ml.) was immediately withdrawn with a sterile 10 ml. pipette, and the tube was allowed to stand for about 2 minutes in order that the ciliates might swim to the top of the remaining milliliter of water. With a sterile 1 ml. pipette, 0.5 ml. of ciliate suspension was withdrawn and placed in an empty sterile centrifuge tube. This suspension was again diluted, and the process repeated until the ciliates had gone through an average of fifteen such transfers with the accompanying dilutions (a dilution factor of approximately 10^{14}). This method entails a great loss of ciliates but was found necessary inasmuch as, without removal to fresh tubes with the consequent discarding of the residue, contamination was invariable. It was demonstrated that the contaminations resulted from the fact that ciliates died or became immobilized during centrifugation and were carried passively to the bottom of the tube with their adhering bacteria. However, by discarding the dead forms we were able to sterilize several hundred ciliates at each attempt, and these gave us the necessary organisms with which to work.

The details of our methods have been given here in the hope that they may prove useful to future investigators. In our opinion it is a fallacy to assume that the removal of bacteria from any free-living protozoan can be accomplished by any one standardized method. Each type will have to be treated individually and its peculiarities studied in order to determine a satisfactory method. And in order to be sure of the end results each step in the manipulation should be checked with bacteriological broth inoculations and poured or flooded (Anderson and Stuart, 1935) agar plates, these being kept for a *minimum of 14 days*. It is only by employing these rigid checks that the slow-growing bacterial contaminants can be detected. All the foregoing precautions and checks were adhered to in our work.

SERIAL TRANSPLANTS IN *Aerobacter* SUSPENSIONS

Starting with sterile ciliates, three series of bacteriological tubes were set up in which were placed 6 ml. of distilled water bacterized with *Aerobacter*. These tubes behaved as did the previous early tubes of the contaminated series, enormous growth of the ciliates with a consequent cleaning-out of the *Aerobacter* taking place within a period of 48 hours, the ciliates then encysting on the walls of the tubes. Just as encystment began, one bacteriological loop of this ciliate culture was transferred aseptically to a fresh tube of each series. Unlike the contaminated series, however, no diminution of ciliate growth took place in the following weeks, and the ciliates did not fail to clear out the fluid. To date these series have been running for over a year through 192 transplants, without showing any decrease in numbers per tube at the end of the 48-hour periods, except in a few instances where the stock *Aerobacter* culture dissociated from the normal smooth form to either rough or mucoid variants. Separate experiments show that neither of these variants of *Aerobacter* is as satisfactory as the normal form.

STERILE CILIATES IN PURE CULTURES OF CHROMOGENIC BACTERIA

Single sterile ciliates were transferred into distilled-water, hanging-drop suspensions of the various chromogenic bacteria which had been isolated from the contaminated tube series, in order to test their specific effects upon growth and reproduction. The hanging-drop method was employed that they might be observed directly.

Along with these experimental slides, controls on the sterility of the ciliates were set up as follows: four or more single ciliates were placed in hanging drops of proteose-

peptone broth and four or more in distilled water. After every tenth experimental setup, broth cultures were again prepared, and this was repeated at the end of the total number. In this way we could check microscopically, in a relatively short period of time, any gross errors in our procedure, as well as the initial sterility of the ciliates. After the experiments were finished, the final check of our drops came from streaking out on plates and examining for possible contaminants. By working in a room devoid of air currents,

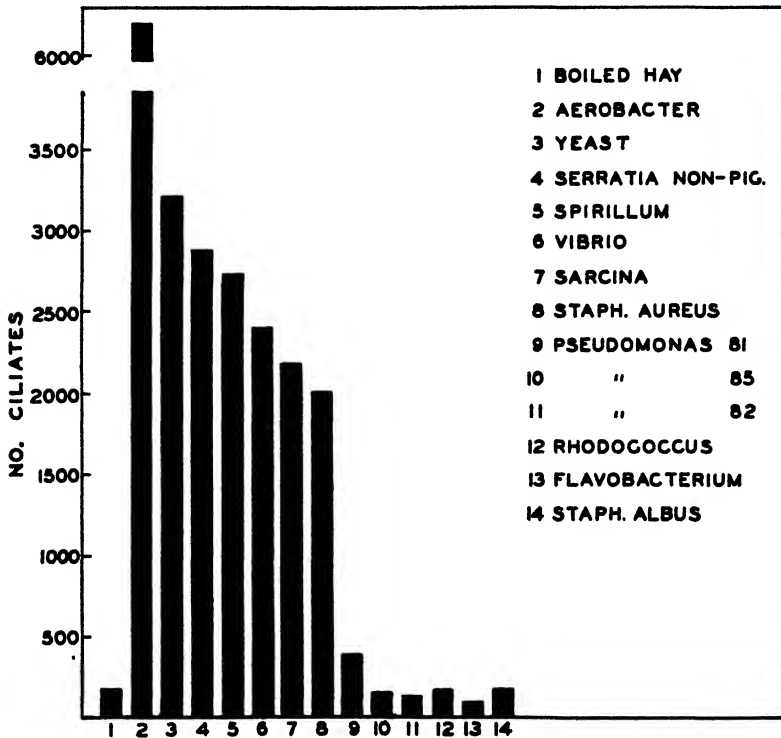


FIG. 2.—Graphic comparison of the total number of cells produced at the end of 48 hours in hanging-drop suspensions of various food organisms. The suspensions were of approximately equal densities (about 10 billion per cc.) at the start of the growth periods. The initial number of *Colpoda* in each case was 1, and the volume of medium was approximately 0.005 ml. All ciliates were completely sterile at the start of the experiments, except No. 1 (boiled hay).

by using only sterile equipment, by performing all manipulations under a Cellophane hood, and by making all transfers under a dust shield covering the stage of the dissecting microscope, we were able to eliminate chance contaminations to less than 1 per cent of the total number of experiments. Those which were found to contain contaminants were so recorded, and the results obtained were discarded.

The chromogens tested can be grouped into three categories in regard to their effects on the ciliates: those which were eaten by the ciliates but produced poor to mediocre growth (as compared to *Aerobacter*); those which were entirely rejected as food but which produced no abnormalities; and those which proved to be toxic.

In the first category the following types were found: *Pseudomonas* (three species designated by us as Nos. 81, 82, and 85), *Staphylococcus aureus*, *S. albus*, *Spirillum rubrum*, *Sarcina lutea*, *Rhodococcus roseus*, and *Flavobacterium* (two species, one orange and one lemon yellow). While the ciliates fed upon these bacteria, in no case did the growth equal one-half that attained when *Aerobacter* was presented as food in comparable concentrations. The relative abilities of these chromogens to support the growth of *Colpoda* can be seen from Figure 2. This figure is, however, misleading as to the end result of the cultures. The numbers given are the number of cells produced at the end of 48 hours, but the culmination of a healthy culture is the formation of resistant cysts. In the case of cultures of *S. albus* and *R. roseus* not only were abnormalities observed in the trophic organisms but all the ciliates died without forming cysts. While cysts were produced in cultures of the three species of *Pseudomonas*, excessive numbers of abnormalities occurred in the form of monsters, a great many of which perished without encysting. In the remaining types, although the growth was limited, encystment always resulted in the normal manner.

In the second category we found only one type, *Flavobacterium* sp. (our orange No. 3). Sterile ciliates placed in suspensions of this bacterium behaved precisely as did those placed in sterile distilled water. In most cases encystment took place after about 36

TABLE 2

BACTERIA NOT SUPPORTING GROWTH

<i>Flavobacterium</i> (orange No. 3).....	Nontoxic
<i>Chromobacterium violaceum</i>	Toxic (trophic and cyst)
<i>Serratia marcescens</i> (pigmented).....	Toxic (trophic and 90 per cent cysts)
<i>Aerobacter cloacae</i> (autoclaved).....	Nontoxic
<i>A. cloacae</i> (killed 100° C.).....	Nontoxic
<i>A. cloacae</i> (killed 55° C.).....	Nontoxic
<i>Serratia marcescens</i> (autoclaved).....	Toxic

hours, the single ciliate having become appreciably smaller. Spontaneous excystment occurred, and the single ciliate, after a period of swimming, would again encyst. Each cyst was smaller than the previous one until, at the end of 6-8 days, the tiny, rounded form would disintegrate. From a careful examination of many ciliates under the highest powers of the microscope we are of the opinion that none of this species of bacteria were ingested by the ciliates. Whatever may be the cause of the rejection of this bacterium, it does offer an explanation for the persistence of this type of *Flavobacterium* in increasing numbers in our contaminated series.

The third category of bacteria proved to be the most interesting from the standpoint of the cultural restrictions. These experiments are presented in Table 2. Sterile ciliates placed in suspensions of pigmented *Chromobacterium violaceum* or *Serratia marcescens* were invariably killed in 2-30 minutes, depending upon the concentration of the bacteria. Both these types are known to produce what has been called a nondiffusible pigment, insoluble in water but soluble in alcohol, chloroform, or ether. It was at first assumed that the toxic effects were produced as a consequence of ingestion of the bacteria by the ciliates, but it was later demonstrated that this could not be the sole explanation. Because of the different effects produced by the two types of bacteria, they will be discussed separately.

In suspensions of *C. violaceum*, taken from an agar slant where the pigment was pronounced, all trophic *Colpoda* exhibited a marked change in their swimming activities.

They became flattened and twisted and moved more and more slowly. Very shortly a distinct lavender flush could be seen throughout the cytoplasm, and motion ceased. Within the space of a few minutes after they became motionless disintegration occurred. This was so striking that large numbers of ciliates from *Aerobacter* tubes were introduced into drop suspensions. The same results were obtained and in about the same time. No protective effect was apparent either from the *Aerobacter* present or from the large numbers of ciliates. Next, large numbers of resistant cysts were placed in drop suspensions of *C. violaceum*. Approximately 99 per cent of these cysts became lavender in color, the color concentrating in the cytoplasm and leaving the cyst wall clear. These cysts showed no protoplasmic activity, and, although they did not disintegrate, they never excysted. The remaining 1 per cent went through a slow but rather normal excystment stage, and the emerging trophic forms were immediately killed.

From the results obtained on the resistant cysts it seems quite clear to us that the toxicity was in some way bound up with the purple pigment of the bacteria. It was also evident that, at least in association with the ciliates, the pigment could and did diffuse out of the bacterial cell, otherwise it could not have made its appearance within the cyst wall. This point was well illustrated when the experiments were repeated, using *Serratia* suspensions.

In suspensions of pigmented *Serratia* trophic ciliates were always killed, as in the previous experiments. The toxicity was manifested in a slightly different way, however, in that the ciliates became rounded but retained their powers of motility. The cytoplasm became more and more pink, the contractile vacuoles became greatly distended, and the ciliates burst very suddenly. When cysts were placed in suspensions of *Serratia* about 90 per cent of them reacted in the same manner as those in suspensions of *Chromobacterium*, except that the cytoplasm became pink at first, then changing to a deep red as if the pigment was being concentrated from the suspension. Here again it appeared that the pigment was diffusing from the bacterial cells and entering the cyst walls. The other 10 per cent remained colorless, excysted in a perfectly normal manner, and upon emergence as trophic ciliates were "immune" to the pigment. This 10 per cent had, in some way, become acclimatized to the pigment and were able to feed and grow upon the *Serratia*. Nor was this acclimatization a matter of cell selection, for very often in double resistant cysts, one sister-cell would become blood red and die while the other sister-cell would remain colorless and would excyst. From these acclimatized ciliates it was possible to start subcultures in heavy suspensions of the bacteria.

It should be noted that the pigment of *Serratia* varies to a considerable degree under different cultural conditions. We have found that *Serratia* grown on nutrient agar produces an orange-red pigment, while those grown on nutrient agar to which 1 per cent glycerine has been added always produce a pink pigment at first, turning to a deep, purplish red. While both of these pigments are toxic, distinct differences in their effects on the ciliates have been noted.

An indication of the answer to the question of pigment as the active toxin was found when sterile trophic ciliates were placed in suspensions of nonpigmented *Serratia*. These were entirely unharmed and were able to feed on these bacteria in a normal manner (see Fig. 2).

That the toxic effect of the pigment is not dependent upon the living *Serratia* and that its toxicity is not destroyed by heat is demonstrated by the fact that autoclaved suspensions of the bacteria proved to be fully as toxic to *Colpoda* as were the live bacteria in suspension.

Recently we have obtained some evidence pointing to a partial explanation of these puzzling phenomena in so far as they relate to the pigment of *Serratia*. It was found that resistant cysts that had been washed in 50-100 ml. of distilled water and then placed in suspensions of pigmented *Serratia* were not killed, but all excysted and then were able to feed unharmed on the bacteria. This would suggest that something is liberated from the trophic ciliate into the medium, capable of acting upon the bacterial cell causing it to liberate its pigment in greater quantity than normal. This high concentration is lethal to all trophics and to 90 per cent of the cysts. This product of the ciliate growth in the medium previous to encystment is presumed to adhere in considerable concentration to the cyst wall. Being soluble, it is removed from the cyst by washing, and the concentration of the pigment outside the bacterial cells remains low during the process of excystment. Upon excystment the ciliates have become acclimatized to the sublethal concentration of pigment normally present in the suspension and, although they presumably secrete more of the substance, are from then on unharmed. The presence of the substance is further demonstrated by placing washed cysts in suspensions of *Serratia* made by using the supernatant fluid from a growing *Colpoda* suspension instead of distilled water. Here the majority of the cysts are killed, as in the case of the unwashed cysts, indicating that enough of the substance is present in the fluid of the growing culture to liberate a lethal quantity of pigment.

It is instructive and interesting to note that similar results were obtained with the nonencysting *Colpidium campylum*. This ciliate, grown bacteria free in a proteose-peptone broth or on the surface of proteose-peptone agar slants, exhibits varying degrees of sensitivity to the toxic action of the pigment of *Serratia*. If a drop of broth is bacterized with pigmented *Serratia* and a few *Colpidium* from a broth culture are introduced, the ciliates are quickly killed, first becoming pink and round. Such is not the case, however, if the *Colpidium* have been removed from an agar slant. They are able to acclimatize themselves to the pigment and grow and reproduce in a normal manner. Now if a broth culture of *Colpidium* is centrifuged, the supernatant fluid used for the *Serratia* bacterization, and agar-grown ciliates introduced into this type of drop, they are all killed.

Here, again, it appears that the ciliates are releasing a substance which may act upon the *Serratia*, causing them to release an abnormally high concentration of pigment. The substance seems to be contained in the broth in which the ciliates have grown. When the ciliates are grown on agar slants, this substance possibly diffuses into the agar and, therefore, would be nearly absent from a loop of these ciliates. But the agar-grown ciliates are nevertheless killed if the substance is provided from a broth-culture centrifugate.

In the case of *Chromobacterium violaceum* suspensions, like experiments yielded similar results but with this difference. Washed cysts of *Colpoda* were found to excyst about 100 per cent, but there followed no acclimatization of the trophic ciliates. All trophic ciliates were immediately killed upon emergence.

THE PIGMENT OF *Pseudomonas*

To revert momentarily to the results obtained with suspensions of *Pseudomonas*, it will be recalled that *Colpoda* grew poorly when feeding on any one of the three species used. What is the relation between the pigment of these types and the pigments of *C. violaceum* and *S. marcescens* in regard to toxicity? We know that the pigment of *Pseudomonas* is diffusible and that bacteria removed from an agar slant leave their pigment behind. Therefore, the ciliates in our experiments would be coming in contact with only a minute quantity of the pigment. To test the activity of concentrated pigment, broth

tubes were inoculated with the three species of *Pseudomonas* used. Within 72 hours a well-defined ring of the blue-green pigment had collected at the top of the tubes. From some of this pigment hanging drops were prepared, and trophic ciliates added to them. They were instantly killed. When cysts were used they, too, were killed. It was found that even in great dilutions the pigments of all three species of *Pseudomonas* were lethal, the length of time before death varying with the concentration. No attempt has been made as yet to determine which fraction of this well-known pigment is responsible for the death of the ciliates. The point of importance for our present consideration is the fact that growth products of such a common soil-and-water form as *Pseudomonas* can produce such a drastic effect upon the ciliates. This observation corresponds in part with that of Cleveland (1928) working with *Tritrichomonas fecalis*. He reported the extreme toxic effects on this protozoan of "exotoxins" of *P. fluorescens* and *P. aeruginosa*.

DEAD BACTERIA AS A SOURCE OF FOOD

In the course of our work we have had occasion to test the possibility of feeding *Colpoda* on dead bacteria. We prepared suspensions of heat-killed *Aerobacter*, killed in three different ways: at 55° C. for 1 hour, at 100° C. for 30 minutes, and autoclaved for 15 minutes. Into hanging drops of these suspensions were placed sterile ciliates. In two cases where *Aerobacter* killed at 55° were used, fair ciliate growth resulted. Both of these drops when plated showed live *Aerobacter*. All other experiments failed to show any ciliate growth and proved to contain no living bacteria when plated. The ciliates behaved in the same manner as those placed in the distilled-water controls. These experiments were repeated on autoclaved, nonpigmented *Serratia* with the same results. In short we have never observed any growth whatsoever in our strain of *Colpoda* in the absence of living food, either in the above-mentioned suspensions of dead bacteria, broths of various types and concentrations, or yeast extracts. The possibility of finding the exact combination of nonliving substances and conditions which will satisfy the nutritional requirements of this ciliate is not ruled out, however, and work is progressing with that object in view.

DISCUSSION

It is unnecessary here to review the work on the sterilization of various species of protozoa inasmuch as this has already been done a number of times before (see Phelps, 1934; Johnson, 1936; Hall, 1937). Space will be devoted, therefore, only to a discussion of the problems relative to the experimental work reported here.

We consider our findings on the ability of extraneous bacteria (many of which are detrimental to ciliate growth) to maintain themselves and increase in numbers in serial transplants to be of significance in any cultural work. We believe that this demonstration alone points out a possible cause of "depressions" in cultures under uncontrolled conditions and may point the way to a revaluation of some of the life-cycle work of the past. It indicates that it is entirely erroneous to assume, because only a few extraneous bacteria are introduced with an organism into a culture medium, although that culture medium may consist of a suspension of a food organism in a non-nutritive fluid, that these few will produce no effect. It is not only probable but inevitable that some form of food selectivity will be exhibited by the experimental animals and that there is always the probability of harmful forms persisting and increasing to a proportion where their effects will be felt. We cannot, therefore, subscribe to any notion of "relative sterility" in preparing protozoa or any other type of bacteria-feeder for experimental use.

It has been pointed out by Chatton and Chatton (1927a; 1927b) that certain chromogenic bacteria (*B. fluorescens* and *B. prodigiosus*, which are our *Pseudomonas* and *Serratia*) exert a cytolytic effect upon *Glaucoma scintillans*, *Colpidium colpoda*, *Colpoda steini*, *C. cucullus*, and *Paramecium caudatum*. They noted that this effect was most pronounced in highly pigmented strains but thought that the toxicity was not due to the pigment itself but was a combination of a glucoside and a lipid. Their experimental evidence upon the nature of the toxic substance is incomplete. It should be noted in this connection that Phelps (1934) was able to grow *P. aurelia* on pure strains of pigmented *Serratia* (*Erythrobacillus prodigiosus*) without noting any harmful effects. There appear to be two possible explanations for these results: (1) that he was able to effect acclimatization in his strain of protozoa to the *Serratia* pigment or (2) that that species of protozoa was not reactive to the pigment. The latter explanation seems the less probable in view of the results obtained by the Chattons on another species of the same genus.

Taylor and his co-workers (Barker and Taylor, 1931, 1933; Thimann and Barker, 1934; Taylor and Strickland, 1935, 1936, 1938; Taylor, Brown, and Strickland, 1936) have contributed a number of interesting reports concerning the growth, encystment, and excystment of *Colpoda*. The species of *Colpoda* used in all these studies should be designated as *C. duodenaria* according to Taylor and Furgason (1938), although it was earlier described as *C. cucullus* and *C. steini*. In these investigations the ciliates were fed on a suspension of *Pseudomonas* in a balanced salt solution with the idea that the bacteria would not increase in numbers during the experiments and that any extraneous bacteria would have no effect because they also would fail to multiply. We have already pointed out that these assumptions are not necessarily correct.

Relative to the question of the ability of *Colpoda* to feed and reproduce by using only dead bacteria as a source of food, we can only harmonize the findings of Oehler (1919, 1920) with our own by the conviction that his ciliates were never entirely free of bacteria when tested. In our experimental setups with dead bacteria we never obtained any growth. Growth resulted in those cultures where, owing to the treatment of the *Aerobacter* (heating at 55° C.), not all the bacteria were killed. Those few grew and were in turn eaten by the ciliates, producing the appearance of growth by feeding on the dead bacteria. In every case, however, where ciliate growth took place, plating disclosed the presence of viable bacteria. The method employed by Cleveland (1928) of growing *Tritrichomonas fecalis* anaerobically on dead bacteria would not be expected to yield results with *Colpoda* when we consider the relatively high oxygen requirements of this ciliate.

It is interesting to note that the food requirements of this strain of *Colpoda* and the reaction of this protozoan to bacterial pigments parallels to a surprising degree those of the cladoceran *Moina macrocopa* (Stuart *et al.*, 1931) and appears to be quite different from those types of ciliates which have been grown in nutrient, bacteria-free broths. This comparison, along with the possession of a mode of reproduction within a cyst wall, seems to point to the classification of *Colpoda* as a highly specialized member of its group.

For the sake of completeness we have included in our Figure 2 a number of organisms tested in a pure state which have not been included in the general discussion. Obviously we have tested only a small proportion of the types of bacteria which would be commonly met with in samples of hay infusions. To test them all would be an impossible task and would probably add little to the value of the work. We feel that this portion of the work demonstrates our points, which are, in summary:

1. Distilled water becomes nutritive for many bacterial types as it becomes the solvent for ciliate metabolites and bacterial disintegration products.
2. Of these many bacterial types some may prove to be deleterious to the ciliates when they reach appreciable concentrations.
3. Deleterious concentrations of bacteria (certain types) may be reached even in serial transplants because of the selectivity of feeding on the part of the ciliates and the accumulated increase in size of contaminant inoculum from transplant to transplant.
4. Certain bacterial pigments are toxic to *Colpoda*, even in low concentrations.
5. *Colpoda* possesses the ability to become acclimatized to the red pigment of *Serratia marcescens*.
6. This strain of *Colpoda* failed to utilize dead *Aerobacter* as food.

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GROWTH STUDIES ON CILIATES. II. THE FOOD FACTOR IN THE GROWTH, REPRODUCTION, AND ENCYSTMENT OF COLPODA¹

(Three figures)

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THE experiments to be reported here were designed to determine quantitatively the potentialities of growth of the ciliate *Colpoda* in distilled water bacterized with the coliform organism *Aerobacter*. The *Colpoda* used were taken from our stock strain, described in Part I of this series (Kidder and Stuart, 1939), maintained on a pure culture of *Aerobacter*. All experiments were performed with aseptic technique, and the same care was taken to prevent contaminations as was outlined in our previous work.

Our earlier experiments showed that when *Aerobacter* alone was presented as food the ciliates exhibited an extremely high reproductive rate. Under a given set of conditions what are the limits of growth of these ciliates and what are some of the factors which operate to determine these limits? Many reports in the literature deal with these questions but, aside from the older isolation culture methods, few have considered them from the standpoint of a single protozoan. We have evidence to show that survival and growth may vary depending upon the initial number of protozoa used. Accordingly, all of our experiments have been started with single trophic ciliates, and our results are designed to demonstrate the potentialities of growth exhibited by these isolated cells under varying conditions of food concentration in a given volume and in varying volumes of a given food concentration. We are making no attempt in this report to analyze our results in relation to possible growth-promoting factors similar to the allelocatalytic agents of Robertson (1921, 1927). Our evidence along these lines will be given later in the series.

Colpoda offers excellent material for investigations of this type because of its ability to form resistant cysts. A culture may be started, and the end result of growth may be determined at any time after growth has ceased, by counting the number of cysts formed. This fact has proved to be of great value where the hanging-drop method is employed. In larger volumes, however, it is impossible to count the cysts directly, but by adding fresh fluid excystment can be induced and the trophics then counted. In other words, the cysts form a permanent record of the growth that has taken place and can be used for counting at any time after growth has stopped.

In addition to the hanging-drop technique we have used sterile 10×40 mm. shell vials containing the varying volumes of bacterial suspensions. With a sterile micropipette one ciliate from stock was introduced into the suspension, and the vial corked with a rubber stopper. It was necessary when the volumes were small to prevent evaporation; so to this end sterile wet cotton plugs were inserted below the rubber stopper to within about 2 mm. of the culture. Each experimental series was set up by tens and repeated three times on successive occasions. Cultures were grown at room temperature (22°–24° C.). The method of counting ciliates was as follows: During the growth period sets of vials were select-

¹ This investigation was supported in part by a grant-in-aid from the Society of the Sigma Xi.

ed, appropriate dilutions of the contents prepared, and the ciliates, in samples of known volume, were counted with the aid of a dissecting microscope. At the end of the growth period when the ciliates had encysted, the fluid was removed and 0.5 ml. of distilled water was added. After excystment was complete, known volume samples were counted, as above. All counts were repeated, and averages taken.

0.005 ML. VOLUMES; VARYING FOOD CONCENTRATIONS

A 1 ml. suspension containing approximately 10 billion *Aerobacter* in distilled water was prepared, and from this suspension six graded dilutions up to 1:100,000 were made. These dilutions were numbered 2-7, from the greatest to the 1:1 (10 billion) series respectively (see legend of Fig. 1). Hanging drops (approximately 0.005 ml. in volume) of each of the six suspensions were prepared in duplicate on sterile cover glasses, and a single ciliate added to each drop. At the end of 48 hours all ciliates had increased in numbers, had exhausted the food supply, and had encysted. By using the mechanical stage of the compound microscope and a hand counter, every cyst in all drops was counted, and the average number taken for each dilution. This experiment was repeated on three successive occasions.

It was found that the average number of ciliates produced in the 1:1 (10 billion *Aerobacter* per ml.) suspension was 6,280 and that the averages decreased as the dilution increased (Fig. 1). In all cases the *Aerobacter* had been removed to a point where none could be detected in the fluid, although a few were still adhering to the cover glass and the surface film. In distilled-water controls (dil. 1, Fig. 1) the ciliates divided so that there was an average of 8 at the end of 48 hours. This multiplication in distilled water is explained by the presence of a few *Aerobacter* introduced with the ciliates (taken from *Aerobacter* cultures) and also by the presence of a certain quantity of unassimilated food within their bodies.

In all the foregoing dilutions the ciliates grew in proportion to the amount of available food (Fig. 1). What would be the result of increasing the concentration of bacteria to a point where all could not possibly be eaten? We prepared hanging drops of distilled water bacterized to the consistency of heavy cream and into these drops placed single ciliates. All the ciliates lived and reproduced at a rate even higher than in the 1:1 suspension. At the end of 48 hours, the time at which the ciliates in the 1:1 suspension encysted, over 20,000 ciliates were present in the "heavy cream" (dil. 8, Fig. 1). Not all the *Aerobacter* were removed, however, and the end was not encystment but sudden rupturing. Just before the ciliates ruptured, the number was so great within the confines of the 0.005 ml. drops that the ciliates were touching one another. We repeated these experiments many times and always with the same results. Obviously we had given the ciliates more food than they could use within such narrow spatial limits. It might be asked, if it were merely a matter of space within which to grow, why, then, after a number of ciliates had disintegrated would not this decrease in protoplasmic bulk allow those intact ciliates to live, feeding on the remaining *Aerobacter*? The answer to this question is that the products of ciliate disintegration are extremely toxic to fresh ciliates and also produce very rapid bacterial agglutination. Into many of these drops were placed from one to hundreds of additional fresh ciliates. These always ruptured within a few minutes.

0.5 ML. VOLUMES; VARYING FOOD CONCENTRATIONS

It appeared from the results of the foregoing experiments that we might gain some valuable information regarding reproductive potentialities if we increased the volume

of the suspensions, thereby increasing the number of bacteria available in lower concentrations. To that end we prepared seven series of vials, each series being a dilution of a

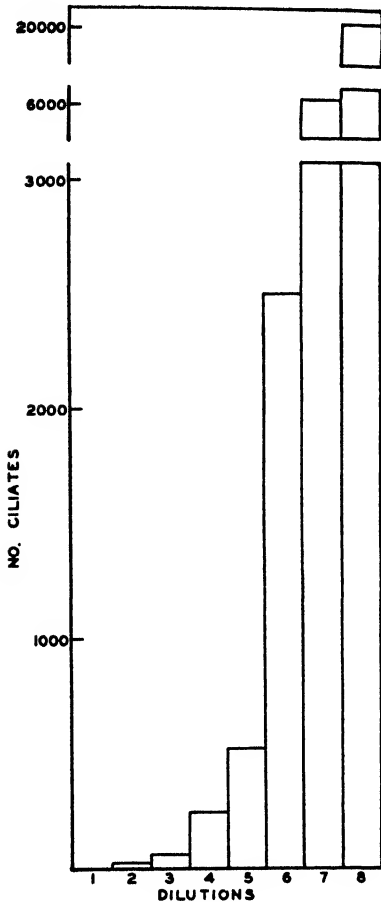


FIG. 1

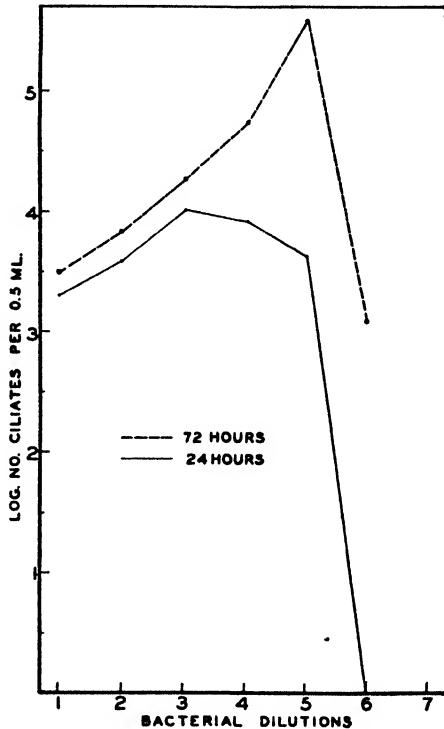


FIG. 2

FIG. 1.—Graphic representation of data obtained from the growth of single ciliates in 0.005 ml. volumes of varying concentrations of *Aerobacter* in distilled water. Direct counts of ciliates made at the end of 48 hours. No. 1 is distilled-water control; No. 7 is a suspension of 10,000,000,000 *Aerobacter* per ml.; Nos. 2-6 are dilutions of No. 7 by tens (No. 2 = 100,000, No. 3 = 1,000,000, No. 4 = 10,000,000, No. 5 = 100,000,000, No. 6 = 1,000,000,000 bacteria per ml.). No. 8 is 0.005 ml. of distilled water bacterized to the consistency of heavy cream. Resistant cysts represented the end result in all dilutions except No. 8, where death occurred.

FIG. 2.—Graphic representation of data contained in Table 1. All points represent averages of 30 vials in 3 successive experiments. Constant volume (0.5 ml.), varying bacterial concentrations (1 = 637,000 per ml.; 2 = 6,370,000 per ml.; 3 = 63,700,000 per ml.; 4 = 637,000,000 per ml.; 5 = 6,370,000,000 per ml.; 6 = 63,700,000,000 per ml.; 7 = 637,000,000,000 per ml.).

heavy suspension of *Aerobacter* (average concentration, for three separate experiments, being 637 billion per ml. viable count). These series were numbered 1-7 from the greatest dilution to the 1:1 series (637 billion), respectively (see legend of Fig. 2). Into each vial

(containing 0.5 ml. of suspension) a single trophic ciliate was introduced. Counts of the numbers of ciliates present at the end of 24 hours were made, and the end result of growth was determined at 72 hours. These results are presented in Table 1 and are graphically represented in Figure 2. In dilutions 1, 2, and 3 there was an increasing difference of reproductive rates at the end of 24 hours. Dilution 4 showed less growth than 3, and dilution 5 was still lower. In most of the vials of dilution 6 the ciliates died, and in the remaining few reproduction was negligible. No ciliate survived in dilution 7, although this concentration was lower than our "heavy cream" bacterized hanging drops. After 72 hours the vast majority of ciliates in dilutions 1 through 5 had encysted, and the fluid was crystal clear. Counts of these end results showed that as the number of available bacteria was increased the number of ciliates produced in a given period of time was greater. In one vial of series 5, 702,000 ciliates represented the end result of the growth of a single cell, and the average of all the vials of that series was 457,620. Series 6 showed only three vials containing living ciliates and no cysts, with the low average count of 1,616.

TABLE 1
0.5 ML. VOLUME; VARYING BACTERIAL CONCENTRATIONS

Dilution Number	Bacteria per Ml.	Ciliates at 24 Hours	Ciliates at 72 Hours	Maximum Number Obtained
1.....	637,000	2,038	3,906	4,940
2.....	6,370,000	3,782	7,338	12,090
3.....	63,700,000	10,070	19,500	22,740
4.....	637,000,000	8,182	61,534	80,900
5.....	6,370,000,000	4,190	457,620	702,000
6.....	63,700,000,000	5 (1 vial)	1,616 (3 vials)	4,850
7.....	637,000,000,000	0	0	0

A number of facts were suggested by these results. If there is sufficient space for growth, the number of ciliates capable of being produced from a single cell in a given period of time varies directly with the number of food organisms present, up to a certain concentration for that volume. The reproductive rate also increases as the food increases up to a certain bacterial concentration. Also the growth-rate of the ciliates is lower in the early stages of the high concentrations, but, as the number of ciliates increases, the growth-rate becomes accelerated so that the peak of the curve in Figure 1 is at dilution 3 at 24 hours but has shifted to dilution 5 at 72 hours. Because concentrations 6 and 7 are lower than our highest concentrations in the hanging drops, it appears that there is a volume effect in these experiments. It must be admitted that physical differences between the vial and hanging-drop fluids might conceivably produce different results which would invalidate any direct comparisons between the two. Comparisons may be made, however, between different concentrations of bacteria in either drops or vials.

VARYING VOLUMES OF A SINGLE FOOD CONCENTRATION

Our next group of experiments was designed to test the effect of varying volumes with a single bacterial concentration. Concentration 7 (637 billion per ml.), in which no single ciliate had lived in the 0.5 ml. volumes, was chosen. Five different volumes were set up

in series of ten vials each, the experiment repeated on three successive occasions. The volumes chosen were 0.01 ml., 0.02 ml., 0.04 ml., 0.08 ml., and 0.16 ml. A single ciliate was introduced into each vial. Counts were made at the end of 36, 48, 60, 72, and 84

TABLE 2
CONSTANT BACTERIAL CONCENTRATION (DIL. NO. 7); VARYING VOLUMES

VOLUME	NUMBER OF CILIATES PER 0.5 ML.					
	36 Hours	48 Hours	60 Hours	72 Hours	84 Hours	Maximum No.
0.01 ml.			669,800	1,090,200		1,680,000
0.02 ml.	5,707	27,900	734,500	1,133,000	1,753,600	2,200,000
0.04 ml.	3,520	24,700	426,500	1,440,000	1,937,400	2,550,000
0.08 ml.	1,300	5,300	23,125	49,330	Dead	204,000
0.16 ml.	60 (1 vial)	0	0	0	0	

hours, except in the case of the 0.01 ml. volumes. The results of these counts are presented in Table 2 and in Figure 3.

Whereas no ciliates survived in this bacterial concentration in the 0.5 ml. volumes in the preceding experiment (Table 1), there was some survival in all the series from 0.01 ml. to 0.16 ml., although in the last series only one vial out of thirty showed growth, and that extremely low.

The smaller volumes showed a higher division-rate through the first 60 hours of growth, but the numbers of ciliates in the 0.04 ml. vials were greater, though only slightly so, at 72 and 84 hours. The relatively small difference in numbers is due to the fact that the end point (encystment) was reached at 72 hours in the 0.01 ml. vials and slightly later in the 0.02 ml., but in the 0.04 ml. vials encystment was not complete even at 84 hours. In the 0.08 ml. volumes the depressing effects noted in the previous experiment occurred, with slow growth up to 72 hours and then rapid death without encystment. Volumes of 0.16 ml. almost completely suppressed growth.

DISCUSSION

We have shown in Part I of this series (Kidder and Stuart, 1939) that many different types of bacteria may exert a growth-limiting influence on a population of *Colpoda* because of their inferior qualities as food or because of their toxicity. When these types are removed and only *Aerobacter* (an excellent food organism) is presented, there occurs an enormous population increase in proportion to the

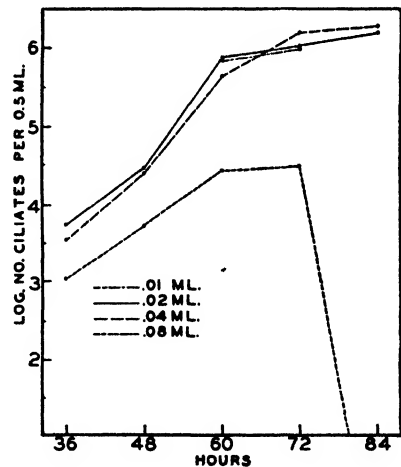


FIG. 3.—Graphic representation of data contained in Table 2. All points represent averages of 30 vials in 3 successive experiments. Varying volumes (0.01 ml.—0.08 ml.), constant bacterial concentration (No. 7 of Table 1, 637,000,000,000 *Aerobacter* per ml.).

concentration of bacteria, within limits. Some of the factors limiting growth under these conditions appear to be the available bacteria, the available space for growth, and the depressing effect of high bacterial concentrations in large volumes. The latter may be due to oxygen deficiency, as has been shown to be the case by Phelps (1936) and Taylor and Strickland (1938) working with larger volumes. In separate experiments we have been able to obtain growth in aeration flasks using larger volumes and heavier bacterial concentrations than could be used without aeration. In the small volumes, however, it appears that the oxygen-tension factor may be of less importance, owing to the large surface area exposed.

Contrary to the earlier views of Woodruff (1911) and a number of others since then, our results show that excretory substances appear to play only a small role in growth limitations of *Colpoda*. This was also the conclusion reached by Phelps (1936) in dealing with bacteria-free cultures of *Glaucoma*.

Crowding—a term applied to conditions where it is thought that the volume of the animals contained in a given space becomes too large for compatibility—operates in the smallest volumes (0.005 ml.) of *Colpoda*, tending to limit and stop their growth. But the crowding here is an actuality not an effect produced by a few organisms in a large volume. The ciliates become so concentrated that they are in actual physical contact with one another. When this condition is reached, the ciliates rupture. We realize that by pointing to physical contact as a limiting factor we have given no explanation but only a description of what happens.

In our opinion a very important factor in the growth limitation of a population of *Colpoda* is the quantity of available food. That other factors enter in, to a lesser degree, we do not deny. Temperature certainly plays a part. Hydrogen-ion concentration may play a small part, but not an appreciable one between the limits of pH 5 and pH 8.5. Decrease in oxygen tension may play a part, especially in larger volumes. Concentration of metabolic waste products of the protozoa or of the food organism is apparently ineffective, otherwise their effects would be seen by the time the ciliates reached the concentration of 20,000 per 0.005 ml.

It seems apparent that the same factors operating to limit growth may influence encystment of *Colpoda*. Dilution 8 ("heavy cream") of Figure 1 shows a concentration equal to approximately 4 million ciliates per ml., and yet no cysts were formed. Thus the view that encystment is induced by an accumulation of metabolic waste products of the ciliates themselves apparently does not apply in the case of this strain of *Colpoda*. This is also the conclusion reached by Taylor and Strickland (1938) in the case of *C. duodenaria*, although it was stated otherwise in an earlier report (Barker and Taylor, 1931).

From our observations we believe that encystment in our strain of *Colpoda* is brought about, under the conditions of our experiments, principally by the lack of available foods and that "crowding" does not result in encystment but in death.

The results here reported do not answer all the questions relating to the interesting question of growth limitations in populations of *Colpoda* but simply point out some of the possibilities under experimental conditions.

SUMMARY

1. The potentialities of growth of a strain of the ciliate *Colpoda* have been investigated.

2. The food used was the coliform bacterium, *Aerobacter*, suspended in distilled water.
3. Single ciliates when introduced into varying bacterial concentrations reproduced in direct relation to the bacterial count.
4. Single ciliates introduced into varying volumes (from 0.01 to 0.16 ml. in vials) of a known bacterial concentration grew readily and to great concentrations in the smaller volumes but practically not at all in the larger volumes.
5. It was concluded that the available food is one of the principal factors operating to limit the population of this ciliate.
6. Encystment appears to be the result of the depletion of the available food, for in no case did it take place when viable *Aerobacter* were present in quantities.

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GROWTH STUDIES ON CILIATES. III. EXPERIMENTAL ALTERATION OF THE METHOD OF REPRODUCTION IN COLPODA¹

(One figure)

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IN ALL species of the genus *Colpoda* reproduction normally takes place within a cyst wall. This wall is secreted by the ciliate during the early phases of mitotic activity and is accompanied by a rounding-out of the cell. Within the division cyst two daughter-cells are produced, and these divide again to produce four cells. There is a gradual redifferentiation of the characteristic morphological structures of the trophic ciliate in each daughter, swimming activities become more and more pronounced until the cyst wall ruptures at one point, and the four daughter-ciliates escape one by one and swim away. On rare occasions two or eight daughter-cells are produced within the division cyst and these escape from the cyst as do the four. This mode of division has been described in detail for a number of species of *Colpoda* and certainly represents the normal method of reproduction.

Alterations in the foregoing method of reproduction do occur, however. Penn (1937) describes quadruple division of *Colpoda cucullus* without the formation of a cyst wall. He is inclined to think that cyst formation is an adaptation on the part of the ciliate to an adverse environment and that quadruple division without a cyst wall represents the normal method of reproduction when the conditions are optimum for growth. This belief has been criticized in regard to *C. cucullus*, since during long-continued cultivation of this species in boiled hay only rare cases of division without a cyst wall occurred (Kidder and Claff, 1938).

Under certain conditions, *Colpoda* sp.² reproduces without a cyst wall. Various other modifications of the normal division-cyst method of reproduction also occur. The conditions under which these departures from the normal make their appearance have been investigated and are the subject for the present report. A few new terms, descriptive in nature, have been introduced.

DESCRIPTION OF THE DIFFERENT TYPES OF REPRODUCTION

In view of the fact that the alterations in the method of reproduction form the basis for what is to follow, a short description of their morphological differences will be given. We have designated the alterations (from the normal "division cyst, DC") as follows: "atypical division cyst" (ADC), "fission-division cyst" (FDC), "fission division" (FD) and "simple fission." Other alterations have been noted, but they do not enter into the present discussion.

Atypical division cyst.—This modification of the normal type of reproduction involves

¹ This investigation was supported in part by a grant-in-aid from the Society of the Sigma Xi.

² The strain of *Colpoda* used in this investigation is the same as that reported in the first paper of this series (Kidder and Stuart, 1939a).

the later stages of the reproductive phase, and it is only at the excystment of the four daughter-cells that it can be detected. During reproduction by normal division cysts, after the four daughters have redifferentiated within the cyst wall, they become very active and resemble in every detail trophic ciliates. When the cyst wall finally ruptures at some point, the ciliates emerge with almost explosive suddenness and immediately swim away. In the atypical division cyst the development of four daughter-ciliates parallels the normal until almost ready to excyst. They are never as perfectly shaped or as active, however, and when the cyst wall ruptures they squeeze out through the resulting opening

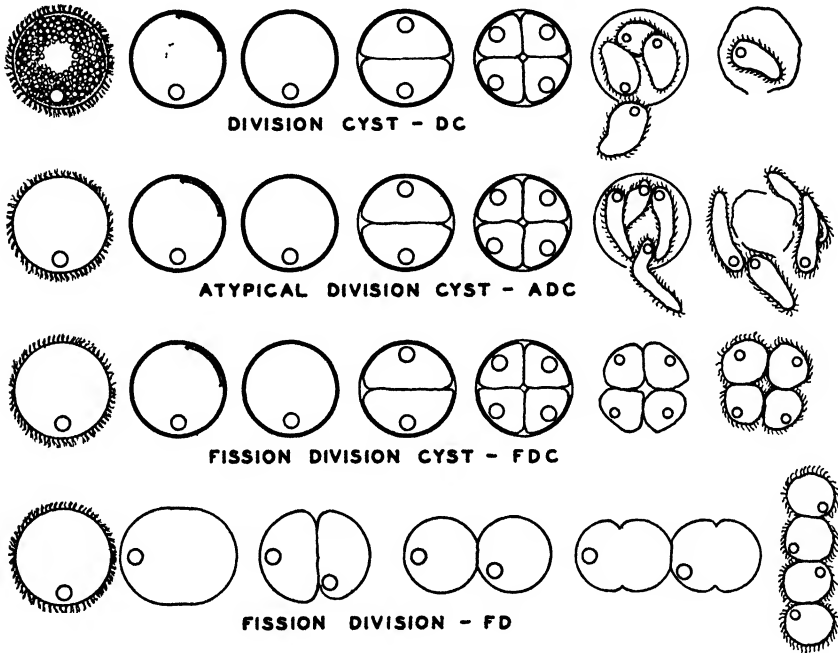


FIG. 1.—Diagrammatic representation of various methods of reproduction in *Colpoda*. Within the cells the dotted circles represent the macronuclei, while the solid circles represent the contractile vacuoles; arrows indicate rotation.

one by one and very slowly. They are always elongated “wormy” forms and do not swim away but remain in the immediate vicinity of the old cyst wall for some time (Fig. 1, atypical division cyst, *ADC*). Eventually they develop normal activity and are indistinguishable from any normal young trophic form.

Here it would seem that the redifferentiation of the daughter-cells is completed outside the cyst wall, since the wall appears to be weak and prematurely ruptures.

Fission division cysts.—Slightly more radical than the foregoing modification is the fission-division cyst. The early stages of this reproductive process are identical with the normal and with the atypical division cyst, but after the four-cell stage is reached and before redifferentiation of the characteristics of the trophic form occurs, the cyst wall ruptures and disappears. This leaves the four daughter-cells exposed to the environment (Fig. 1, fission-division cyst, *FDC*). Slow redifferentiation takes place and finally the four round,

feebly moving ciliates become somewhat separated. Eventually each attains the normal form and swims away.

In this modification the cyst wall is weaker and less resistant than in the previous one, and shortly after it ruptures no trace of it can be seen.

Fission division.—This type of reproduction is accomplished without the formation of a cyst wall. As the ciliate prepares for reproduction it rounds up in the normal manner. The revolving motion, so noticeable in the early stages of the normal division cyst, is entirely lacking. For that reason fission division can be detected in the very early phases. The cilia appear to be partially withdrawn, and motion ceases. Slight elongation takes place during the period of macronuclear division, and the first plane of fission ensues. The two daughter-cells become quite round but do not separate. Each daughter elongates and divides, the planes of their division being parallel to the first fission plane instead of at right angles to it, as in the division cyst. This results in a characteristic chain of four cells, all rounded and retaining contact with each other (Fig. 1, fission division, *FD*). The cilia become active, and the whole chain vibrates slowly. Eventually, the four daughters separate, slowly attain the normal form, and start swimming.

It should be noted, in relation to the second fission plane, that its direction, parallel to the first, is apparent but not real. Before the second fission starts there is rotation on the part of the first two daughters, so that the polarity is probably not different from the normal division-cyst daughters.

This type of reproduction appears to be approaching the condition found in the vast majority of ciliates where no cyst is formed. It is characteristically quadruple fission, although occasionally only one division occurs.

Simple fission.—Under certain conditions, rare cases of irregular binary fission have been observed. Elongation of a swimming trophic ciliate occurs, and eventually two daughter-ciliates result. The fission plane is somewhat oblique to the long axis of the body. The body outlines of both daughters are irregular and appear somewhat abnormal, but when isolated these daughters are found capable of normal growth, reverting immediately to the division-cyst type of reproduction.

With the occurrence of simple fission we see that this ciliate is capable of altering its method of reproduction from its normal highly specialized division cyst, through a series of changes, to the binary fission type characteristic of most ciliates.

EXPERIMENTAL PROCEDURES AND RESULTS

On January 28, 1938, a $5'' \times \frac{5}{8}''$ culture tube containing 6 ml. of sterile distilled water was bacterized with a 24-hour agar slant culture of *Aerobacter cloacae*. In all experiments herein reported this species was used exclusively for bacterization purposes. Several *Colpoda* (Kidder and Stuart, 1939a) from a hay infusion culture were inoculated into the bacterial suspension. A week later this tube was rebacterized with approximately the same amount of bacteria. At this time the usual heavy ring of resting cysts had formed on the tube at the surface of the liquid. Thereafter, the tube was rebacterized at 7- to 10-day intervals, and sterile distilled water added to keep the contents of the tube at about the same level. By the third or fourth bacterization the ring of resting cysts had completely disappeared, and so far as could be ascertained resting cysts did not again form in this tube during the course of the experiment, which continued for approximately 10 months.

The ciliates originally inoculated into the experimental tube carried over some of the

bacterial flora of the hay infusion. It seemed of interest to determine whether the extraneous bacteria would survive the frequent massive inoculations of *Aerobacter*. Survival had been shown to occur in 48-hour serial transplants (Kidder and Stuart, 1939a). Accordingly, flooded plates were prepared at different intervals throughout the experiment from suitable dilutions of the suspension from the experimental tube. These plates indicated that the proportions of the extraneous bacterial flora remained relatively constant throughout the experiment. Four to five days after bacterization with *Aerobacter* the organisms present in the tube were roughly estimated as 80 per cent *Pseudomonas* (it should be noted that while the diffusible pigment of the *Pseudomonas* organisms was very marked on the agar plates it was never observed in the experimental tube), 15 per cent *Flavobacterium*, and small numbers of *Aerobacter*, *Achromobacter*, *Bacillus*, *Clostridium*, and other undetermined genera.

At irregular intervals bacterized hanging drops containing from 3 to 5 ciliates were prepared from the liquid in the experimental tube. As a control similar preparations were made from a 24-hour tube of our *Colpoda-Aerobacter* pure series (Kidder and Stuart, 1939a). In this way the growth of the ciliates could be observed in a medium in which growth products had accumulated for only 24 hours, as well as in a medium in which growth products were constantly accumulating. The speed of growth and number of divisions in the experimental and control hanging-drop preparations were approximately equal for 6 months. After 6 months, growth of ciliates in the experimental tube steadily declined. The growth of experimental ciliates inoculated into hanging drops of bacterized distilled water also steadily declined. In October, 1 ml. of the contents of the experimental tube had to be concentrated by centrifugation in order to obtain ciliates, while in November, approximately 10 months after the start of the experiment, no ciliates were found when the entire contents of the tube was concentrated.

From January 28 to May 1 no change in the method of reproduction was noted in the ciliates from the experimental tube. On June 28, however, hanging drops prepared from the experimental tube showed that 100 per cent of the ciliates were dividing by fission division. Control preparations of normal ciliates from S 42 (the forty-second transplant of ciliates which had been sterilized and were being cultivated on pure *Aerobacter*) showed only regular division cysts.

On June 30 both the ciliates and the ciliate-free medium of the experimental tube were studied. One milliliter of the medium was centrifuged at 2,000 r.p.m. for 6 minutes to concentrate the ciliates. About 0.75 ml. of the supernatant fluid was then transferred to a second tube and centrifuged at high speed to remove all ciliates. The supernatant fluid from this second tube was saved, and the centrifugate discarded. To the concentrated ciliates in the first tube 10 ml. of distilled water were added, and the tube centrifuged again at low speed. Approximately 9.5 ml. of the supernatant fluid were discarded and 9.5 ml. of distilled water added. This procedure was repeated five times to dilute out accumulated growth products.

Unless otherwise specified all experiments were set up in duplicate.

Loopfuls of the ciliate-free supernatant fluid from the experimental tube were heavily, moderately, and lightly bacterized, single washed ciliates from the experimental tube were added to these suspensions, and the cover glasses inverted and sealed onto depression slides. This procedure was repeated with the washed ciliates in distilled water bacterized as above. Washed normal ciliates from S 43 were set up in the same way in the supernatant fluid of the experimental tube and also in distilled water. The results of these experiments are recorded in Table 1.

TABLE 1

WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON JUNE 30 IN
BACTERIZED EXPERIMENTAL SUPERNATANT FLUID

Bacterization	Experiment	Type of Reproduction*
Heavily.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 7G</i> —complete rupture <i>FD 7G</i> —complete rupture
Moderately.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 6G—DC 7G—RC</i> many degenerate <i>FD 5G—DC 6G—RC</i>
Lightly.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 3G—DC 4G—RC</i> <i>FD 3G—DC 4G—RC</i>

WASHED EXPERIMENTAL CILIATES IN BACTERIZED DISTILLED WATER

Bacterization	Experiment	Type of Reproduction*
Heavily.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 7G</i> —complete rupture <i>FD 7G</i> —complete rupture
Moderately.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 5G—DC 6G—RC</i> <i>FD 5G—DC 6G—RC</i>
Lightly.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 4G—DC 5G—RC</i> <i>FD 3G—DC 4G—RC</i>

WASHED NORMAL CILIATES IN BACTERIZED EXPERI-
MENTAL SUPERNATANT FLUID

Bacterization	Experiment	Type of Reproduction*
Heavily.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 7G</i> —complete rupture <i>FD 7G</i> —complete rupture
Moderately.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 6G—DC 7G—RC</i> some degenerate <i>FD 5G—DC 6G—RC</i>
Lightly.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>FD 3G—DC 4G—RC</i> <i>FD 4G—DC 5G—RC</i>

WASHED NORMAL CILIATES IN BACTERIZED DISTILLED WATER

Bacterization	Experiment	Type of Reproduction*
Heavily.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>DC 7G—RC</i> <i>DC 7G—RC</i>
Moderately.....	$\begin{Bmatrix} 1 \\ 2 \end{Bmatrix}$	<i>DC 6G—RC</i> <i>DC 6G—RC</i>
Lightly.....	1	<i>DC 4G—RC</i> <i>DC 4G—RC</i>

* *FD*—Fission Division; *RC*—Resting Cyst; *DC*—Division Cyst; *G*—Generation.

Example: *FD 6G—DC 7G—RC* many degenerate. Ciliate reproduced by fission division for six generations, by division cysts in the seventh generation. Resting cysts were then formed, many of which were degenerate.

Table 1 shows that ciliates from the experimental tube in both heavily bacterized supernatant fluid and distilled water reproduced for seven generations by fission division then ruptured. In the moderately bacterized suspensions the ciliates reproduced by fission division for five to six generations, then for one generation by division cysts, finally forming resting cysts. In the lightly bacterized preparation fission division extended to the third or fourth generation, followed by one generation of division cysts, before resting cysts were formed. It will be noted that where the concentration of food is not too heavy a division cyst always intervenes between fission division and resting cysts. Over 500 such preparations have been followed, and in no case have resting cysts been observed to form immediately after a generation of fission division. In several experiments cover slips were removed, and fresh unbacterized supernatant fluid added at the fourth or fifth division; nevertheless, a generation of division cysts occurred before resting cysts were formed. It cannot be said that occasional individual ciliates do not form resting cysts immediately after fission division, since, where we are dealing with hundreds or thousands of ciliates in one drop, it is impossible to follow each ciliate. Ciliates in unbacterized experimental supernatant fluid produced one or two generations by fission division, then a generation by division cysts followed by resting cysts. The total number of ciliates is sufficiently small that it can be safely said that no resting cysts formed except after a generation of division cysts. Even washed fission-division forms in unbacterized distilled water produced one generation by division cysts before going into resting cysts.

Washed normal ciliates in bacterized experimental supernatant fluid reproduced by fission division just as did the experimental ciliates. On the other hand, normal ciliates in bacterized distilled water produced only regular division cysts. This would seem to indicate that the medium in the experimental tube had been altered or conditioned by the growth of the ciliates, by the growth of the bacteria, or by both. For convenience the term "conditioned medium" will be substituted hereinafter for "the supernatant fluid from the experimental tube," and, when referring to what seems to be some substance or substances in the conditioned medium responsible for certain reactions, the term "conditioning factor" will be used.

There were at this time (June 30), 21,000 ciliates per milliliter in the experimental tube. Distilled water was added to the experimental tube, and bacterization continued until November 16.

On July 28, August 28, October 4, and November 16, the reaction of normal and experimental ciliates to the conditioned medium, the species specificity of the conditioned medium, the nature of the conditioned medium, and other factors were studied. The results of these experiments will be discussed under various headings.

Normal ciliates in conditioned medium.—Normal ciliates from S 55 in moderately bacterized conditioned medium on July 28 produced, as in the previous month, 100 per cent fission division for five to six generations, then one generation of division cysts followed by resting cysts. Ciliates from S 55 in bacterized distilled water reproduced by division cysts. The rate of division of the normal ciliates in the conditioned medium was slower than in the distilled water and, moreover, small numbers of degenerate resting cysts were formed in the conditioned medium, and only normal resting cysts in the distilled water.

The same results were obtained with ciliates from S 68 on August 28 and from S 85 on October 4, except that the rate of division in conditioned medium was progressively slower than the controls in distilled water. Ciliates in the conditioned medium of October 4 formed, for the most part, degenerate cysts.

On November 16, ciliates from S 104 in the conditioned medium reproduced by fission division for only two to three generations. After that division cysts, fission-division cysts, fission division, and atypical division cysts all occurred in some hanging drops. While these ciliates produced from six to seven generations in 96 hours, the trophic forms ruptured without forming resting cysts. Thus the conditioning factor which was first found in the experimental tube on June 28 was present but only in weak concentration on November 16. The fact that no ciliates were found in the experimental tube at this time may account for the weakness of the conditioned medium. The controls, ciliates in S 104 in bacterized distilled water, produced six to seven generations by division cysts in 48 hours, and formed normal resting cysts.

It was noted that the first division of a normal ciliate in conditioned medium might be either by division cysts or by fission division. The type of division seemed to be determined by the time elapsing before division. Ten normal ciliates immediately after excystment, ten ciliates 2 hours after, and ten ciliates 3.5 hours after excystment were individually placed in bacterized conditioned medium. The first ten ciliates (immediately after excystment) all reproduced by fission division. Six of the next group reproduced by fission division and four by division cysts. All of the last group reproduced by division cysts. The succeeding five or six generations in every case were produced by fission division.

To determine the effect of continued growth of a normal ciliate in conditioned medium, duplicate hanging-drop preparations were made, on July 28, of ciliates from S 57 in bacterized conditioned medium. The ciliates reproduced by fission division for six generations, then one generation of division cysts followed by resting cysts. Just before resting cysts were formed, a ciliate was isolated from each preparation and again set up in conditioned medium. This procedure was repeated ten consecutive times. Each transfer produced results wholly comparable to the first setup. The ciliates from the tenth transfer, before forming resting cysts, were washed five times in distilled water by centrifugation, and the isolated ciliates set up in bacterized distilled water. These ciliates reproduced the same as they had in conditioned medium. They were again washed and set up in bacterized distilled water. Fission division occurred for only three generations, followed by one generation of mixed fission-division and atypical division cysts, two generations of fission-division cysts and division cysts, and one generation of division cysts only. A third transfer of washed ciliates produced only division cysts.

It would seem from this that a normal ciliate growing in conditioned medium for approximately sixty-five generations continues to reproduce by fission division for several more generations when transferred to bacterized distilled water. It will be shown later that the conditioned medium of July 28, when diluted ten times, did not cause fission division in normal ciliates. Since the material from the tenth transfer was diluted more than a million times by washing, it is improbable that sufficient conditioned medium could have been carried over into the bacterized distilled water to cause fission division. It is also difficult to conceive of sufficient conditioned medium, adsorbed on the surface of the isolated ciliate, being carried over to the distilled water to cause fission division for seven generations.

Ciliates from the experimental tube in distilled water and in conditioned medium.—On June 28, the first time the conditioning factor was found in the experimental tube, washed single ciliates from this tube, in bacterized distilled water, reproduced by fission division, for five to six generations, then by division cysts for one generation, followed by resting cysts. Washed ciliates from the same tube on July 28 showed marked variation in meth-

ods of reproduction in bacterized distilled water (Table 2). The ciliates in Experiment 4 (Table 2) reacted as had all the ciliates the previous month. The ciliates in Experiment 8 reacted in reverse fashion, producing five generations of division cysts, one of fission division, and finally a generation of division cysts. On the other hand, ten washed ciliates from the experimental tube in bacterized conditioned medium produced only fission-division forms for five or six generations, followed by the usual generation of division cysts.

Serial transfers of the ciliates in Experiments 4 and 8 (Table 2) to bacterized distilled water were carried out in the manner previously described. Ciliates from Experiment 4 continued to reproduce by fission division to about the sixth generation for six consecutive transfers, to the fourth generation on the seventh, and to the second generation on

TABLE 2
WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON JULY 28 IN
BACTERIZED DISTILLED WATER

Experiment	Type of Reproduction*
1	<i>FD</i> 4 <i>G</i> — <i>ADC</i> + <i>FDC</i> + <i>DC</i> 5 <i>G</i> — <i>DC</i> 6 <i>G</i> — <i>RC</i> many degenerate
2	<i>FD</i> 5 <i>G</i> — <i>DC</i> 6 <i>G</i> — <i>RC</i>
3	<i>FD</i> 5 <i>G</i> — <i>DC</i> 6 <i>G</i> — <i>RC</i>
4	<i>FD</i> 6 <i>G</i> — <i>DC</i> 7 <i>G</i> — <i>RC</i> many degenerate
5	<i>DC</i> 3 <i>G</i> — <i>ADC</i> 4 <i>G</i> — <i>FDC</i> 5 <i>G</i> — <i>FD</i> 6 <i>G</i> — <i>DC</i> 7 <i>G</i> — <i>RC</i> many degenerate
6	<i>FD</i> 4 <i>G</i> — <i>DC</i> 7 <i>G</i> — <i>RC</i>
7	<i>FD</i> 7 <i>G</i> complete rupture
8	<i>DC</i> 5 <i>G</i> — <i>FD</i> 6 <i>G</i> — <i>DC</i> 7 <i>G</i> — <i>RC</i> many degenerate
9	<i>FD</i> 3 <i>G</i> — <i>FDC</i> + <i>ADC</i> + <i>DC</i> 5 <i>G</i> — <i>DC</i> 6 <i>G</i> — <i>RC</i> few degenerate
10	<i>FD</i> 6 <i>G</i> — <i>DC</i> 7 <i>G</i> — <i>RC</i>

* *DC* = Division Cysts; *ADC* = Atypical Division Cysts; *FDC* = Fission-Division Cyst; *FD* = Fission Division; *RC* = Resting Cyst; *G* = Generation.

Example: *FD* 4*G*—*ADC* + *FDC* + *DC* 5*G*—*DC* 6*G*—*RC* many degenerate. Ciliates reproduced by fission division for four generations. In the fifth generation some of the ciliates reproduced by atypical division cysts, others by fission-division cysts, and some by division cysts. In the sixth generation all ciliates reproduced by division cysts. Resting cysts were then formed, many of which were degenerate.

the eighth transfer. The next transfer showed only regular division cysts. Ciliates from Experiment 8 reproduced by division cysts for two generations in the second transfer, then by fission division to the sixth generation. In the next three transfers reproduction was by fission division with a return to reproduction by division cysts on the next transfer. Thus after a month or more in conditioned medium, several consecutive transfers in bacterized distilled water were required before normal division cysts were again produced. We cannot account for the fact that the ciliates in Experiment 8 first produced division cysts, followed by fission division, which in turn were followed by division cysts.

On August 28, washed ciliates from the experimental tube were again set up in bacterized distilled water and in bacterized conditioned medium. The ciliates in the conditioned medium reproduced by fission division as in previous experiments. The reactions of the ciliates in distilled water are shown in Table 3. All the ciliates reproduced by division cysts for at least four generations. Some then produced fission-division forms, others did not. A ciliate from Experiment 1 (Table 3) was carried through two transfers in

bacterized distilled water. In both transfers reproduction was by fission division to the fifth or sixth generation. A ciliate from Experiment 3 reproduced by division cysts for two consecutive transfers. In the third transfer reproduction occurred by division cysts

TABLE 3
WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON AUGUST 28
IN BACTERIZED DISTILLED WATER

Experiment	Type of Reproduction*
1.....	DC 4G—ADC+FD 5G—FD 6G—DC 7G—RC
2.....	DC 5G—ADC+FD 6G—DC 7G—RC
3.....	DC 7G—RC
4.....	DC 5G—ADC+FD 6G—DC 7G—complete rupture
5.....	DC 6G—DC+ADC+FD 7G—RC+rupture+degenerate RC
6.....	DC 7G—RC
7.....	DC 5G—FD 6G—DC 7G—RC some degenerate
8.....	DC 6G—RC
9.....	DC 5G—ADC+FD 6G—FD 7G rupture
10.....	DC 5G—ADC+AFD+FD 6G—DC 7G—RC

* For explanation see Table 2.

to the third generation, then by fission division to the sixth generation. Reproduction by fission division for five generations took place in the fifth transfer.

Washed ciliates from the experimental tube in bacterized conditioned medium reproduced by fission division on October 4. The reactions of ciliates in bacterized distilled

TABLE 4
WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON
OCTOBER 4 IN BACTERIZED DISTILLED WATER

Experiment	Type of Reproduction*
1.....	DC 5G—DC+FD 6G—DC 7G rupture+degenerate RC
2.....	DC 6G—RC
3.....	DC 7G—RC many degenerate
4.....	DC 5G—DC+FD 6G—FD 7G rupture
5.....	DC 7G—RC many degenerate
6.....	DC 7G—RC few degenerate
7.....	DC 6G—RC
8.....	DC 6G—RC
9.....	DC 7G—RC many degenerate
10.....	DC 7G—RC few degenerate

* For explanation see Table 2.

water are recorded in Table 4. It will be noted in this table that only ciliates 1 and 4 produced any fission-division forms. Ciliates from Experiments 2, 7, and 10 were carried through seven transfers. Growth of the ciliates was increasingly poorer in the second, third, and fourth transfers. An average of only four generations was produced in the fourth transfer. Small numbers of fission-division forms (not more than 10 per cent)

were produced in each series in the fourth or fifth generation of the third to fifth transfers. All other reproduction was by regular division cysts.

It has been shown that washed ciliates from the experimental tube on June 28 reproduced by fission division in bacterized distilled water. On July 28 some of the ciliates from the same tube reproduced by fission division, others by division cysts. The latter reproduced by fission division in subsequent transfers in bacterized distilled water. All washed ciliates from the experimental tube on August 28 produced only division cysts for from four to seven generations. Selected ciliates in serial transfers reproduced entirely by fission division in one or another transfer. On October 4 only two of the ten ciliates tested reproduced by fission division. Serial transfers of those producing only division cysts produced no more than 10 per cent fission division in any generation of any transfer. Had ciliates been found in the experimental tube on November 16, it is possible that they would have failed to reproduce by fission division under any condition. At the present time no explanation for these facts is apparent.

Species specificity to the conditioned medium.—To test the species specificity of the conditioned medium, fifteen wild *Colpoda* were isolated from several infusions of soil or grass in water. These ciliates were individually transferred to tubes containing 6 ml. of bacterized distilled water. After they had become acclimatized and had multiplied in this environment, single ciliates (in duplicate preparations) were placed in hanging drops of bacterized distilled water and in bacterized conditioned medium. Nine of the strains reproduced by division cysts, in the conditioned medium and in distilled water. Five serial transfers of these ciliates were made in bacterized conditioned medium without any change in their method of reproduction. Upon structural analysis all these nine strains were found to differ from our stock strain of *Colpoda*.

Ciliates from the six remaining cultures, Nos. 3, 6, 7, 8, 9, and 12 in bacterized conditioned medium reproduced by fission division for five or six generations as did our stock strain from S 59. In bacterized distilled water all, with the exception of strain 3, reproduced only by division cysts. Strain 3 in the fifth generation showed atypical division cysts and two fission-division forms. These six wild strains and ciliates from S 59 were transferred serially for from four to seven transplants in bacterized distilled water. (It should be pointed out that where a number of strains were to be transferred into bacterized distilled water or conditioned medium, the required amount of liquid was bacterized before loops of the material were placed on the cover slip to receive the ciliate, in order to insure equal amounts of food.) Strain 3 showed about 10 per cent fission division in the fourth and fifth generations of the second transfer; about 20 per cent in the third to fifth generations of the third transfer; and about 50 per cent in the fourth generation of the fourth transfer. At this point all the ciliates of this strain ruptured. Strains 6, 7, 8, 9, 12, and S 59 showed only division cysts in seven transfers, but marked variation in the reproduction rate and the total number of divisions were noted. Stock ciliates from S 59 and strains 7 and 9 produced from six to seven generations in 48 hours, culminating in normal resting cysts. Strain 6 produced only four to five generations in 72 hours with many degenerate resting cysts. Strains 8 and 12 produced five to six generations in 48 hours with normal resting cysts.

Strains 6, 7, 8, 9, and 12 were found to be identical with our stock strain of *Colpoda* on the basis of the number of ciliary rows, position and shape of the mouth, and type of nuclei, both resting and fission. Strain 3, although the ciliary lines, mouth, and macronucleus are identical with our stock, differs in one important detail. The micronucleus is

extremely small. The number of chromosomes appears to be large, while eight is the number for our stock strain, (The evidence for this will be reported at a later date.)

These strains have been carried in the laboratory for 5 months. Strains 8 and 12, in the usual 6 ml. of bacterized distilled water, produced in 48 hours a heavy ring of cysts about the tube; strains 7 and 9 grew well but sometimes failed to encyst; while Nos. 3 and 6 frequently had to be transferred to 0.5 ml. of bacterized distilled water in a small vial (Kidder and Stuart, 1939b) to insure maintenance of the strains. It is evident from this that not all strains of the same species of *Colpoda* are alike in their growth potentialities, and as is the case with bacteria, one should hesitate before assuming that the reactions obtained with a single strain of a species will necessarily hold true for all strains of that species even under identical conditions.

Reactions of a modified strain of the stock Colpoda.—In the course of sterilizing cultures of *Colpoda* as previously described (Kidder and Stuart, 1939a) loops of the washed ciliates were inoculated into tubes containing 6 ml. of sterile broth as a check on sterility. One such tube which showed no evidence of growth after 7 days was reincubated. After 14 days a ring of resting cysts was noted, but the broth appeared clear. The broth was plated out and found to contain a pure culture of a *Mycobacterium* which requires 9 days for the production of pin-point colonies. As the ciliates were growing in a pure culture of the *Mycobacterium*, transplants were made at 2- or 3-week intervals into broth and the culture designated as *M* ciliates. It has been since found that this represents an almost ideal way for carrying this strain of *Colpoda*. No bacterization is necessary, the bacteria never overgrow the ciliates, and transplants at 2- or even 3-month intervals are all that are required.

The *M* series was carried in a special rack, and old transplants were not discarded. The first transplant was made on March 26. By August 28 the broth had evaporated to less than 1 ml., yet there was a large number of very small trophic ciliates in the medium. On that date the usual hanging-drop preparations in bacterized conditioned medium and bacterized distilled water were made from the *M* ciliates of the March 26 and the August 12 tubes.

Ciliates from the March 26 tube reproduced very slowly by division cysts in both conditioned medium and distilled water. After the fourth generation the ciliates were large and filled with vacuoles. After five generations all the ciliates ruptured. Before all had ruptured, however, some ciliates from the conditioned medium were transferred to fresh bacterized conditioned medium, and some from distilled water to fresh bacterized distilled water. Reproduction in both media was again by division cysts and through six generations. When rupturing began to occur at this point, a third transfer of ciliates to their respective media was made. In the third transfer the organisms in conditioned medium reproduced for three generations by division cysts, one generation by atypical division cysts and two generations by fission division. In the distilled water reproduction was by division cysts. Four more transfers were carried out. In the conditioned medium there was fission division only for five or six generations when the usual division cysts appeared, followed by resting cysts. In the distilled water only division cysts were produced. It will be noted that all ciliates in these experiments came from the original stock strain, yet a drastic modification of their environment rendered them nonsusceptible to the action of the conditioned medium until after the fourteenth generation.

Ciliates from the August 12 *M* series reacted quite differently. In bacterized conditioned medium reproduction was by fission division only for six generations, followed by

division cysts and resting cysts. In bacterized distilled water, however, five fission-division forms were observed in the seventh generation. Seven consecutive transfers were made to bacterized distilled water. Fission division steadily increased until on the seventh transfer it was 100 per cent. All the ciliates on the two (duplicate) cover glasses were then transferred to a tube with 6 ml. of bacterized distilled water. This tube was re-bacterized every 48 hours. Reproduction, as seen microscopically in loops of the tube's contents, continued by fission division. At 2-day intervals 0.5 ml. quantities of the liquid were centrifuged at high speed and tested for the conditioning factor. Each 0.5 ml. was replaced by 0.5 ml. of distilled water. The conditioning factor first appeared in 16 days (produced some fission division in the fifth generation of S 76) and in 20 days was sufficiently concentrated to produce 100 per cent fission division (normal concentration) for six generations of S 78. A loop from this tube was inoculated into a fresh tube of bacterized distilled water and was treated in the same manner. The conditioning factor in normal concentration was demonstrated in 9 days. The experiment was continued until, in a fresh transplant, the conditioning factor was produced in sufficient quantity in 24 hours to induce 100 per cent fission division in the third or fourth generation of the stock strain. In 48 hours, however, the conditioning factor was present in normal concentration.

Nature of the conditioning factor.—The concentrations of the conditioning factor in the experimental tube on July 28 and on August 28 was determined with ciliates from S 55 and S 68 respectively. Ciliates were placed in bacterized concentrated (1:1), and bacterized dilutions (distilled water) of conditioned medium (1:2.5, 1:5, 1:10, 1:50, and 1:100). In the concentrated and the 1:2.5 dilution, reproduction was by fission division to the sixth generation; in the 1:5 dilution, to the third or fourth generation; no fission-division forms were found in the 1:10 or higher dilutions. At one time some of the *M* ciliates produced conditioned medium of sufficient concentration to be effective in a dilution of 1:10, but not 1:25. This was the strongest conditioned medium obtained.

When relatively large amounts of conditioned medium were made available by the *M* ciliates, 1 ml. was evaporated to dryness at atmospheric pressure, 1 ml. *in vacuo* over sulphuric acid, and each taken up in 0.2 ml. of distilled water. Both preparations were effective in producing fission division in normal ciliates, but approximately half of the potency of the conditioned medium had been lost in drying. Five milliliters of the conditioned medium was reduced to 0.5 ml. *in vacuo*. The concentrated material was capable of causing fission division, but again was about one-half as potent as the unconcentrated substance. When the concentrated material was diluted ten times, it produced no effect on the ciliates. An actual destruction or alteration of the conditioning factor seems to occur both in drying and in concentrating.

The potency of the conditioned medium was impaired by heating to 50° C. for 5 minutes and completely destroyed or inactivated at the same temperature for 10 minutes.

All attempts to precipitate the conditioning factor by alcohol failed. Berkefeld filtrates of conditioned medium lacked the conditioning factor. When powdered Keeselgoehr was added to the conditioned medium, then removed by high-speed centrifugation, the supernatant fluid was also devoid of the active principle. This would seem to indicate that the conditioning factor was not removed by filtration per se, but by adsorption on the electro-negative particles of which the Berkefeld filter is composed.

On July 28 a small amount of conditioned medium was set aside at room temperature in a sealed ampule. Weekly, for 7 weeks, the ampule was opened, tested for the active

principle, and resealed. For 4 weeks the activity of the conditioned medium was unimpaired, a slight decrease was noted by the fifth week, and a marked decrease by the sixth week. By the seventh week the conditioning factor had completely disappeared. Conditioned medium stored in a rubber-stoppered tube at 2° C. depreciated only slightly in 2 months.

Confirmatory production of conditioned medium by the method first described.—On June 30 stock ciliates (S 42) were placed in 6 ml. of bacterized distilled water in four tubes. These tubes were bacterized at weekly intervals, each tube receiving the growth on a 24-hour agar slant culture. At the time these tubes were set up, single ciliates from S 42 were placed in four hanging drops of bacterized distilled water. After the sixth generation the ciliates from each drop were transferred to each of four tubes containing 5 ml. of bacterized distilled water. Thirty-six hours later the total contents of each of these last tubes were transferred to each of four bottles containing 45 ml. of bacterized distilled water. This procedure is necessary since single ciliates do not ordinarily survive in large volumes of medium (Kidder and Stuart, 1939b). The bottles, in contrast to the tubes, had to be bacterized at 2- or 3-day intervals with approximately one-half of a slant culture to insure survival of the ciliates.

Three of the four tubes and one of the four bottles showed the presence of the conditioning factor in weak concentration on October 4, producing a few fission divisions in the fourth, fifth, or sixth generation of stock ciliates (S 85). On November 16, the conditioned medium from three of the tubes produced 100 per cent fission division of S 104 ciliates. The conditioning factor was present in the other tube and in all the bottles but in lower concentrations.

DISCUSSION

It is well known that marked changes are sometimes produced in the medium in which organisms are growing. In some cases the changes are detrimental to the subsequent growth of the same or other species, as evidenced by lowered division and increased death-rates. In other cases the conditioned medium seems to increase the division-rate and lower the death-rate. Changes in the oxidation and reduction potential, in the hydrogen-ion concentration, in the food supply, in the concentration of metabolic products, and many other factors, doubtless play a part in altering the medium. In the present work none of these factors has been studied. In fact it is not known whether the ciliates or the bacteria are responsible for the effects noted in our experiments. There is, however some evidence which tends to show that the ciliates, more than the bacteria, condition the medium. In the original experimental tube approximately 5 months elapsed before the ciliates reproduced by fission division and before the medium was conditioned to the point where it would cause normal stock ciliates to reproduce by fission division. Strain 3, from the wild, reproduced by fission division after several transplants to bacterized distilled water. Unfortunately, no attempt was made to condition medium with this strain. A strain of stock ciliates which had been reared in a pure culture of *Mycobacterium* produced fission-division forms in a single transplant to bacterized distilled water and was later able to condition fresh bacterized distilled water in 24 hours so that the conditioned medium caused normal stock ciliates to reproduce by fission division. Since at the time fission division occurred in these three strains of ciliates the bacterial flora was very similar, it is possible that the previous environment of the ciliates had more influence than

the bacteria in conditioning the medium. Furthermore, the effect of the conditioned medium appears to be species specific.

It is possible that the conditioning factor arises spontaneously in the ciliate as might an adaptive endo-enzyme in response to one or more environmental factors. Under this condition only the ciliate in question and its progeny are affected. If the environment continues to be suitable for the production of the conditioning factor, it may be liberated into the medium, eventually reacting on all ciliates of the same species.

From the preceding evidence it is obvious that the method of reproduction in *Colpoda* is subject to alterations according to certain changes in the environment of the ciliate. A single *Colpoda* destined to reproduce by a division cyst can be made to undergo fission division in conditioned medium. Other types of reproduction have been encountered, such as the atypical division cyst. In this type there appears to be premature release of the ciliates some time after the cilia form, due possibly to a weak cyst wall. In fission-division cysts the entire cyst wall ruptures before cilia have formed. In fission division no cyst wall is formed at all. It might seem that the conditioned medium merely exerts a lytic action on the wall of the division cyst. If such action does take place, it cannot account for all the reactions of these ciliates in conditioned medium. In strong conditioned medium ciliates produce one generation of regular division cysts before going into resting cysts. This occurs even when more conditioned medium is added a generation or two before the division cysts are to be formed. In bacterized conditioned medium 6 generations are usually produced by fission division before division cysts occur, while in unbacterized conditioned medium division cysts followed by resting cysts occur after the second generation in the same concentration of conditioned medium. Ciliates placed in conditioned medium about one hour before reproducing form perfectly normal division cysts. Ciliates placed in conditioned medium for 1 to 2 hours immediately after normal division and then washed free of conditioned medium, reproduce by fission division. Furthermore, as already pointed out, ciliates from *M* series reproduced in bacterized distilled water for many generations by fission division before the conditioning factor was demonstrable in the medium in which the ciliates were growing. It is difficult to escape the conclusion that the conditioning factor acts on some internal factor or mechanism in the ciliates causing a change in their method of reproduction.

As previously pointed out, this strain of *Colpoda* has been observed to reproduce in several different ways. Moreover, when the conditions are right, the various types of reproduction do not occur haphazardly but in orderly sequence. In the proper concentration of conditioned medium a ciliate has been observed to reproduce by a regular division cyst. The next generation reproduced by atypical division cysts, in which the cyst wall seems weak. The next generation reproduced by fission-division cysts, in which the cyst wall ruptures as soon as the second division within the cyst is completed. This was followed by a fission division, in which no cyst wall is formed and in which there is some question as to whether the cilia are completely lost during the division. In fission division numerous cases have been observed wherein a second division did not occur and the two individuals developed quite normally. Finally, eighteen cases have been carefully followed in which the ciliates reproduced by simple fission, while continuing to swim about in the medium. The evolutionary implication of this train of events is clear, but for the present must remain only an implication.

CONCLUSIONS

1. *Colpoda* may reproduce in a variety of ways, and under certain conditions the type of reproduction can be experimentally controlled.
2. It has been demonstrated that conditioned medium can be used to induce different types of reproduction in this strain of *Colpoda*.
3. The conditioning factor is unstable, thermolabile, cannot be concentrated, and is adsorbed on a Berkfeld filter. It appears to be species specific.

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THE VITAMIN B COMPLEX AND THE GROWTH OF COLPIDIUM STRIATUM¹

(Five figures)

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EARLIER attempts to determine the limiting factors for growth of *Colpidium striatum* in bacteria-free cultures indicated that small quantities of some substance or substances found in yeast extract, partially hydrolyzed casein, and liver infusion were responsible for the success or failure of a medium to support continued growth of this ciliate (Hall and Elliott, 1935). Traces of these substances were essential for continued growth of this protozoan in such incomplete proteins as zein, gliadin, and gelatin, even though the missing amino acids were replaced. This fact led to the inevitable conclusion that some factor or factors of the vitamin B complex were involved. Therefore, "pantothenic acid," a universally occurring substance similar to vitamin B₃ (Williams *et al.*, 1933) was investigated and found to have an accelerating effect on the growth-rate of this protozoan (Elliott, 1935). Since pantothenic acid has not been identified as a specific vitamin, this investigation was planned to study the role played by certain purified members of the vitamin B complex in the nutrition of *C. striatum*. The factors investigated were thiamin chloride, riboflavin, and a vitamin B₆ concentrate.

MATERIAL AND METHODS

The experimental organism employed in this investigation was *C. striatum*, a ciliate protozoan isolated by the author in pure-line, bacteria-free cultures several years ago (Elliott, 1933). This organism is definitely mammal like in its food requirements, since it thrives only on complex nitrogen and carbon sources (Elliott, 1935*a*, 1935*b*). For experimental purposes the protozoa were cultured in 250 cc. pyrex flasks, and frequent bacteriological tests assured complete freedom from contamination with other micro-organisms. The basic culture medium used throughout the studies, with the exception of Series I, was composed of the following substances:

NaCl.....	0.003 gm.	FeSO ₄	Trace
KNO ₃	0.002 gm.	Tryptone.....	20.0 gm.
CaSO ₄	0.020 gm.	Dextrose.....	2.0 gm.
MgSO ₄	0.005 gm.	Glass-distilled H ₂ O. . .	1.0 liter

The solution exclusive of the tryptone and dextrose is spoken of as "artificial tap water." For experimental purposes large quantities of the basic medium were prepared in pyrex flasks, one-half of which were rendered vitamin B-free in order to determine the exact effect of the vitamin tested. Heretofore, research work on the nutrition of protozoa usually involved a study of the accelerating effect of the substance in question on the

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growth-rate of the organism in a medium already adequately provided with food requirements. This method failed to indicate whether or not the tested substance was essential in the nutrition of the experimental organism. In the present investigation the vitamin tested was removed from the medium and subsequently replaced by the purified product which indicated whether or not it was an essential growth factor for *Colpidium*. The vitamin B complex was destroyed in the basic medium by adjusting the pH to 9.6 and then heating in the autoclave for one hour at 20 pounds pressure. The pH was then readjusted to 5.8-6.2 before final sterilization. Experiments showed that with this method growth in the treated basic medium was negligible in the first transfer and ceased altogether in the second, indicating that most of the vitamin B complex had been destroyed. Tests for vitamin B₁ with 2,6-dibromoquinone-chloroimide according to the method of Raybin (1938) were negative. Vitamin-free casein was also employed as an alternative method but was unsatisfactory for two reasons: (1) It was necessary to digest the casein with pepsin before the ciliates were able to utilize it, and this involved added complications. (2) It was discovered by several experiments that thiamin was present in sufficient quantities to permit continued growth which impaired the validity of the results. Therefore, the heat treatment was relied upon throughout most of the experiments.

Once the basic medium was rendered vitamin B-free and the pH adjusted to approximately 6.0 which is optimum for *Colpidium*, it was dispensed to 250-cc. flasks in 100 cc. quantities. The vitamin was added to the medium which was again autoclaved, this time for sterilization only which required 15 pounds for 15 minutes. The flasks were then inoculated with the ciliates which had previously been thoroughly washed according to the following procedure. A rapidly growing culture in the untreated basic medium was centrifugalized and the supernatant fluid decanted off; this process was repeated several times with artificial tap water as the diluent. The protozoa were then added to a 250-cc. flask containing 100 cc. of sterile artificial tap water. From this inoculating flask the organisms were transferred by means of graduated pipettes in measured amounts to the test flasks. The inoculation flask was always thoroughly shaken before each transfer in order to insure equal numbers of ciliates in each inoculum. The entire process involved aseptic technique, and frequent bacteriological tests indicated that contaminations were rare; all contaminated cultures were eliminated from the experiments. By this procedure a negligible amount of the original stock culture medium was transferred to the test flasks, which assured reasonably accurate results on the first series of transfers; however, several successive transfers were always made in order to confirm the effect observed in the initial one. The test flasks were incubated in a constant temperature bath at $26^{\circ} \pm 0.02^{\circ}$ C. The amount of growth was determined by means of the protozoa-ocrit method (Elliott, 1939). The initial inoculations were estimated and found to be uniform in all cases. Estimations of growth were made at regular intervals, usually 12 or 24 hours, throughout the growth period of the cultures. It was found quite satisfactory to make estimations on living samples which consisted of 10 cc. of the culture withdrawn aseptically from the test flasks. Duplicate and triplicate cultures were always maintained, and where differences varied more than 5 per cent the series was repeated in its entirety.

The thiamin chloride used in this investigation was obtained in 0.01-gm. ampoules from Merck and Company; the vitamin-free casein, crystalline riboflavin, and vitamin B₆ concentrate from the S.M.A. Corporation; the tryptone, isoelectric casein, and gelatin from the Difco Laboratories; and the 2, 6-dibromoquinone-chloroimide from the East-

man Kodak Company. The vitamins were prepared in solution shortly before use in the tests to insure maximum activity. The initial and final pH determinations were made colorimetrically.

THIAMIN CHLORIDE

Series I.—In view of previous efforts to culture *C. striatum* on incomplete proteins, it was thought worth while in this initial series to attempt to grow this organism on one complete protein (isoelectric casein), one incomplete protein (gelatin), and one partially hydrolyzed protein (tryptone, an enzymatic casein digest), supplementing each with thiamin chloride. Half of the basic medium was adjusted to pH 8.8 and treated with heat in the autoclave for 30 minutes at 15 pounds pressure. This heat treatment was later shown to be inadequate to destroy all the vitamin B₁. The pH was then adjusted to 6.0 in the treated media and distributed to six flasks for each protein; a similar set of flasks contained the control media which were identical with the test media except that during the heat treatment the pH was retained at 6.0. To one-half of the flasks of both the control and the test media was added thiamin chloride making a final concentration of 1:500,000. The flasks were then autoclaved for sterilization and inoculated with approximately equal numbers of washed ciliates. They were then incubated in the constant-temperature bath for 6 days. The volume of protozoa was then estimated for each flask by means of the protozo-o-crit.

It was obvious from the results of this series (Fig. 1) that none of the flasks containing gelatin and casein supported growth to any appreciable extent; furthermore, there was no evidence of any effect of the added thiamin. The heating at a high pH seemed to have rendered the gelatin even less suitable for growth than the untreated product. Transfers to a second series of flasks resulted in no growth at all. It is apparent that thiamin chloride is not the limiting factor for growth when gelatin and casein are employed as basic proteins. However, the results with tryptone, a partially hydrolyzed casein product, are quite different. The heat treatment seemed to have had little effect in destroying the vitamin B₁, since growth is nearly as good in the untreated medium. The supplemented thiamin brought about accelerated growth to a significant degree in the treated culture. On the other hand, the untreated tryptone supported equal growth with and without the addition of thiamin—a fact which indicates that there was sufficient thiamin in the original tryptone to support excellent growth. However, it is not clear why growth in the treated tryptone plus vitamin B₁ was better than in the untreated tryptone supplemented with thiamin.

The results of this series indicate that thiamin chloride is not the limiting growth factor in cultures employing whole casein or gelatin as a source of nitrogen, although partially hydrolyzed casein responds to the vitamin, providing heat treatment has occurred.

Series II.—In this series the heat treatment was made more drastic by extending the time of autoclaving to one hour and raising the pressure to 20 lbs. and the pH to 9.6. Twelve flasks were prepared, six of which were treated to remove the vitamin B complex; the other six were autoclaved along with the first, the only difference being that the pH was set at 6.0 instead of 9.6. Thiamin chloride was added to one-half of the treated and to one-half of the untreated flasks in a concentration of 1:100,000. The remaining flasks were retained as controls. Inoculation and incubation were performed as in Series I, and estimations of growth were made at 12-hour intervals. The growth record (Fig. 2) indicates two distinct facts: (1) The vitamin was almost completely destroyed in the

treated medium by the heating process at pH 9.6. (2) The supplemented thiamin was definitely the limiting growth factor. This last fact was indicated since growth was almost as good in the treated tryptone plus thiamin as it was in the untreated protein. Addition of vitamin B₁ to the untreated tryptone had no effect whatever, indicating that this protein possesses sufficient thiamin naturally to support maximum growth of *C. striatum*.

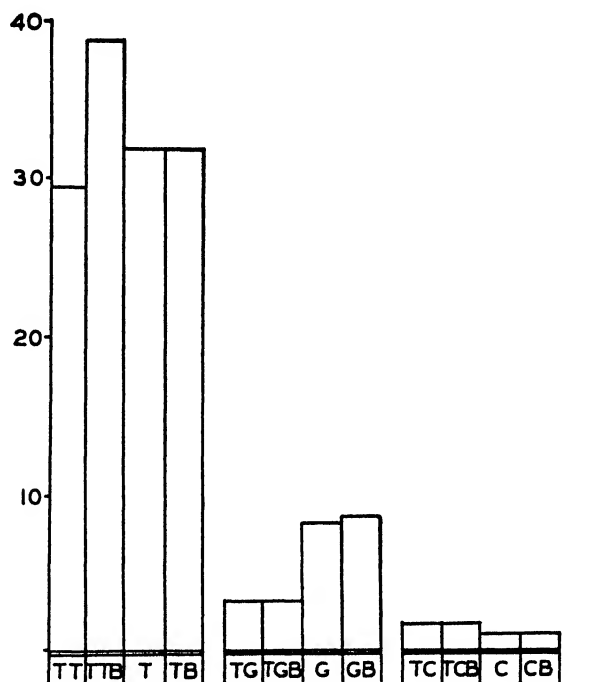


FIG. 1.—Series I. Volumes of packed ciliates in cubic millimeters plotted for the various proteins which are indicated as follows: TT=heat-treated tryptone; TTB=heat-treated tryptone plus thiamin; T=untreated tryptone; TB=untreated tryptone plus thiamin; TG=heat-treated gelatin; TGB=heat-treated gelatin plus thiamin; G=untreated gelatin; GB=untreated gelatin plus thiamin; TC=heat-treated casein; TCB=heat-treated casein plus thiamin; C=untreated casein; CB=untreated casein plus thiamin. There is a statistical-significant difference between TT and TTB; not between TTB, T, and TB.

In order to prove that vitamin B₁ is absolutely essential for growth of this ciliate, it was necessary to continue the cultures through several transfers. This was done, and it was demonstrated that growth after the eighth transfer was equally as abundant as in the first. Therefore, it is concluded that thiamin chloride is essential in the nutrition of *C. striatum*.

EFFECT OF DIFFERENT CONCENTRATIONS OF THIAMIN CHLORIDE

Since it was clear that the limiting factor for growth in the previous series was thiamin, it was necessary to determine which concentrations supported optimum growth and whether or not excessive amounts were injurious.

Series III.—In this series 18 flasks were prepared with heat-treated basic medium, 100 cc. to each flask. The thiamin chloride was added to the flasks in the following concentrations: 1:10,000; 1:100,000; 1:1,000,000; 1:10,000,000; 1:1,000,000,000. The flasks were run in triplicate with three controls containing no vitamin. Inoculations and incubation were carried out as usual, and samples were taken at 24-hour intervals after the first day of growth; two samples were taken during the fourth day of incubation. The

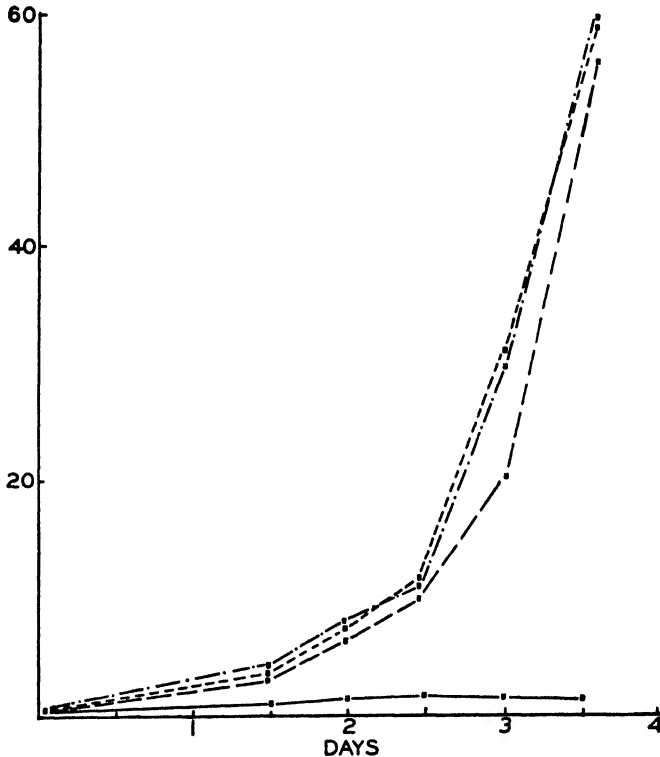


FIG. 2.—Series II. Volumes of packed ciliates in cubic millimeters plotted against time in days. The various media are indicated as follows: — heat-treated tryptone; — — — heat-treated tryptone plus thiamin; - - - - - untreated tryptone; · · · · · untreated tryptone plus thiamin.

record (Fig. 3) indicates the same sort of effect observed in Series II. It is clear that a vitamin B₁ concentration of 1:1,000,000,000 was insufficient to support optimum growth. Concentrations above this seemed adequate at the 84-hour period of growth, but concentrations of 1:10,000 produced the best results. When growth continued another 12 hours, the flasks with concentrations of 1:100,000 and 1:10,000 fell off in their growth-rate, while those with concentrations of 1:1,000,000 and 1:10,000,000 continued to support superior growth. Additional experiments, not recorded here in detail, with concentrations as high as 1:2,000 of thiamin showed no better growth than those with 1:10,000,000; on the other hand, no injurious effects were noted with such high concentrations. It is concluded, therefore, that concentrations of thiamin chloride ranging from 1:10,000 to 1:10,000,000 are sufficient to support excellent growth.

RIBOFLAVIN

Even though thiamin chloride seemed to be the limiting factor for growth in the basic medium of the previous series, it was thought worth while to determine whether or not crystalline riboflavin—vitamin B₂—might be able to replace thiamin in the nutrition of *C. striatum*. For that reason Series IV was conducted.

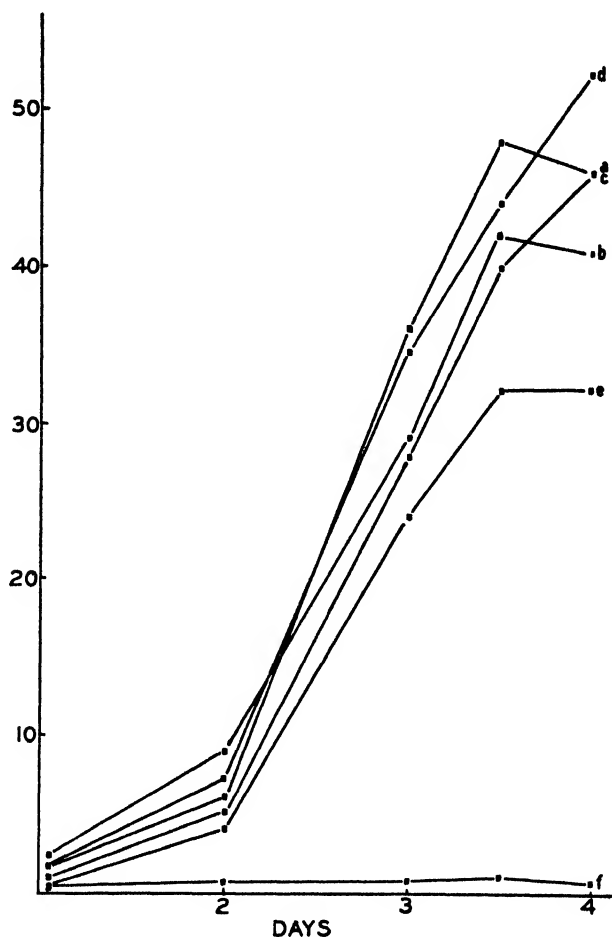


FIG. 3.—Series III. Volumes of packed ciliates in cubic millimeters plotted against time in days. The concentrations of thiamin are indicated as follows: *a* = 1:10,000; *b* = 1:100,000; *c* = 1:1,000,000; *d* = 1:10,000,000; *e* = 1:1,000,000,000; *f* = control with no thiamin.

Series IV.—Sixteen flasks of heat-treated basic medium were prepared, each containing 100 cc. of medium. Crystalline vitamin B₂ was added to these flasks in the following concentrations: 1:2,500; 1:25,000; 1:250,000; 1:2,500,000; 1:25,000,000; 1:1,000,000,000. The series were run in duplicate. Two flasks containing no riboflavin and two containing thiamin chloride in concentrations of 1:100,000 were maintained for comparative

purposes. Sterilization, inoculation, and incubation were conducted as usual. Estimations of growth were made at 24-hour intervals after the second day of growth. The record (Fig. 4) demonstrates that growth was maintained at a low level for the first five days in flasks containing concentrations of 1:2,500, 1:25,000, 1:250,000, and 1:2,500,000; growth was no better than the control in the two most dilute flasks, so no record was

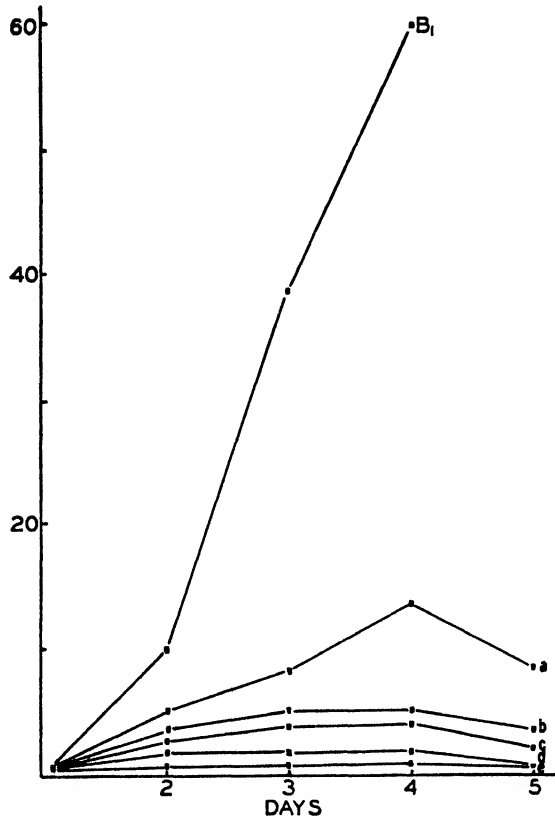


FIG. 4.—Series IV. Volumes of packed ciliates plotted against time in days. The concentrations of riboflavin are indicated as follows: a = 1:2,500; b = 1:25,000; c = 1:250,000; d = 1:2,500,000; e = control with no riboflavin; B_1 = thiamin.

made. The optimum growth in the flasks containing thiamin was reached on the fourth day. While growth was supported at a very low level with riboflavin at certain concentrations, it was clear that this might not continue through several successive transfers. In order to check this point transfers were made, and it was found that growth was just perceptible in the second transfer and ceased altogether in the third. Parallel tests with digested vitamin-free casein indicated similar results. Cultures containing thiamin in addition to riboflavin gave no better growth than those with vitamin B_1 alone. It was concluded, then, that riboflavin is incapable of replacing thiamin chloride in the nutrition of *C. striatum*.

VITAMIN B₆

Even though riboflavin appeared to be unnecessary in the nutrition of *Colpidium*, it is possible that certain other members of the vitamin B complex might possess the properties necessary to replace vitamin B₁. For that reason a concentrate containing vitamin B₆ was investigated.

Series V.—The procedure in this case was similar to that employed in Series IV. Fourteen flasks of treated basic medium were prepared, to which the following concentrations

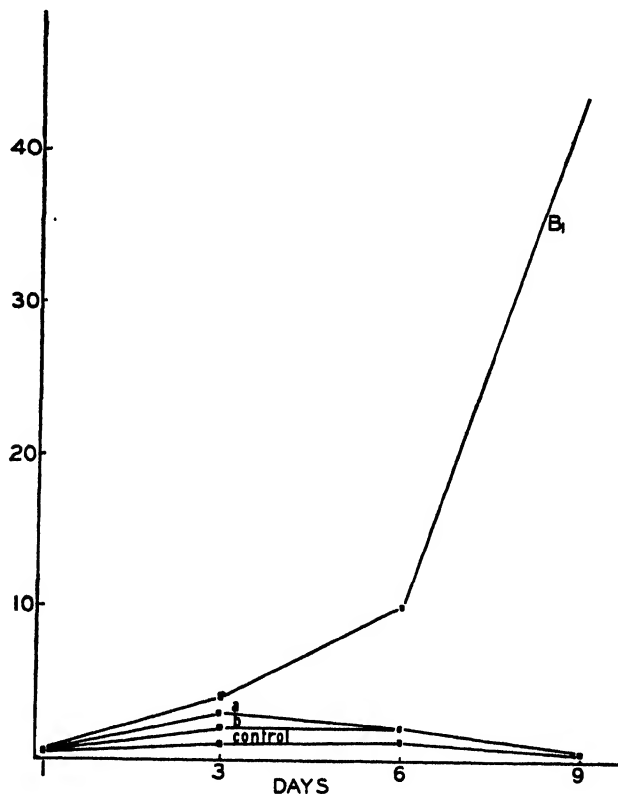


FIG. 5.—Series V. Volumes of packed ciliates plotted against time in days. The concentrations of vitamin B₆ are indicated as follows: *a* = 0.1 cc.; *b* = 2.5 cc.; *control* = no vitamin B₁; B₆ = thiamin.

of vitamin B₆ were added: 0.1 cc. to the first pair of flasks, 0.5 cc. to the second, 2.5 cc. to the third, 5 cc. to the fourth, and 15 cc. to the fifth. Flasks receiving smaller quantities than 15 cc. were supplemented with proper additions of artificial tap water. Two controls were run, as well as two flasks containing thiamin (1:100,000) for comparative purposes. Sterilization, inoculation, and incubation were conducted as in previous series. Samples were taken and estimated on the third, sixth, and ninth days of incubation. The record (Fig. 5) indicates that growth, as compared with the controls, was slightly stimulated in flasks containing concentrations of 0.1 cc. and 2.5 cc. Concentrations higher than these inhibited growth altogether and therefore were not recorded. For compari-

son, growth in the flasks containing thiamin demonstrated a long-lag phase but were normal otherwise. Transfers to a second set of flasks demonstrated complete cessation of growth at all concentrations. Tests with basic medium supplemented with thiamin and riboflavin as well as vitamin B₆ concentrate, showed no better growth than those with thiamin alone. It is concluded then that the factors existing in the vitamin B₆ concentrate employed in this investigation were unable to replace B₁ in the nutrition of *C. striatum*.

DISCUSSION

The fact that certain substances, now recognized as probable members of the vitamin B complex, influence the growth of micro-organisms has been a well-known fact for many years. Wildiers (1901) obtained his so-called "bios" from yeast which had a marked effect on the growth-rate of the same organism from which it was derived. Later, Williams *et al.* (1933) isolated a universally occurring substance, "pantothenic acid," which had a pronounced effect on the growth of yeast and more recently on *C. striatum* (Elliott, 1935) and was thought to be closely related to vitamin B₂. Crystalline thiamin chloride (vitamin B₁) has recently been shown to be essential in the nutrition of many plants and animals. Even such simple forms as propionic acid bacteria require this substance or certain of its decomposition products (Tatum, Wood, and Peterson, 1936). It is also demanded by *Staphylococcus aureus* (Knight, 1937). Isolated pea and tomato roots require thiamin in their normal growth (Bonner, 1937; Robbins and Bartley, 1937). It is essential in normal insect development (Trager and Subbarow, 1938), and its role in mammalian nutrition is well known. Vitamin B₁ apparently plays an important role in the normal nutrition of all plants and animals so far investigated.

Because of its universal need the writer expected that a similar demand would be found among the protozoa. Hall and Elliott (1935) suspected that certain members of the vitamin B complex might be the limiting factor or factors for growth of the ciliate, *C. striatum*; this thought was further suggested when it was learned that pantothenic acid caused a marked acceleration of growth (Elliott, 1935). Later it was demonstrated and reported in abstract form (Elliott, 1937) that crystalline vitamin B₁ played an important part in the growth of this ciliate. Likewise, Lwoff and Lwoff (1937), working with a closely related ciliate, *Glaucoma piriformis*, and Lwoff, M. (1937), with a flagellate, *Strigomonas oncopelti*, showed a similar effect. However, certain protozoa like *Polytoma obtusum* require neither part of the thiamin molecule (Lwoff and Dusi, 1937). Both *Glaucoma* and *Strigomonas* require the intact molecule which is also true of certain plants as well as of mammals. While the pyrimidine and thiazole portion of the thiamin molecule were not investigated in this series of experiments, it is highly probable that the requirements of *Colpidium* would be little different from those of *Glaucoma*.

The fact that thiamin was not the limiting factor in cultures containing gelatin (Series I) was not surprising in view of the recent report by Hall (1938), showing that pimelic acid made possible the successful culture of *C. campylum* in a gelatin medium containing dextrose. This fact does not preclude thiamin as an essential growth factor for *C. campylum*, since no effort was made to control this phase of the experiment; it merely demonstrates that pimelic acid is necessary for continued growth of this ciliate in gelatin.

Earlier experiments (Elliott, 1933) demonstrated clearly that *C. striatum* elaborates enzymes capable of coagulating casein in litmus milk, but it has never been possible to culture this ciliate on pure unhydrolyzed casein, although it has been grown for several

years in the casein enzymatic digest, tryptone. It would appear that the ciliate is incapable of producing enzymes which will hydrolize this protein. The supplemented thiamin in Series I made no difference in the matter of utilization of casein by the ciliate.

Vitamin B₂ (lactoflavin or riboflavin) is an essential factor in the diet of mammals (Ansbacher *et al.*, 1936), as well as of other animals. For instance, Trager and Subarrow (1938) have shown that not only thiamin but also riboflavin is essential for the normal development of the mosquito larva, *Aedes aegypti*. The role played by this vitamin among the protozoa is unknown. It was obvious from the results of this investigation (Series IV) that riboflavin could not replace thiamin in the nutrition of *C. striatum*. This was an anticipated result, because all the vitamin B complex was supposed to have been destroyed with the heat treatment, and the supplemented thiamin was adequate to support abundant growth through eight successive transfers. It is possible that the heat treatment failed to destroy all the vitamin B₂, and a sufficient amount remained to permit excellent growth such as occurred in Series I, II, and III where thiamin was the only added vitamin. However, parallel experiments with so-called vitamin-free casein revealed similar results. Here, again, perhaps all the lactoflavin was not removed from the casein. Many commercial brands of vitamin-free casein have been shown to contain traces of lactoflavin (chemically identical to riboflavin) (Supplee *et al.*, 1936). It is interesting that this ciliate which is so mammal like in all other food requirements can subsist without vitamin B₂. The present series of experiments in no way prove this point; they merely show that riboflavin cannot replace thiamin. Other more detailed experiments are being conducted at present to discover, if possible, the exact role of riboflavin in the nutrition of *C. striatum*.

There is nothing known about the effect of vitamin B₆ on the growth of protozoa. The present investigation shows clearly that this vitamin will not replace thiamin in the nutrition of the ciliate studied. Hutner (1936) has shown that *Euglena gracilis* requires "a water soluble, thermostable material widely distributed in nature and present in relatively high concentrations in autolyzed yeast and urine." According to a series of experiments not reported here, this factor is not thiamin; it may be riboflavin or vitamin B₆. It is possible that not all the vitamin B₆ was destroyed in the heat treatment and therefore remained in small quantities in the basic medium; then when vitamin B₁ was omitted from the medium in Series V, growth was impossible. However, growth in Series I, II, and III would be very good because sufficient vitamin B₆ would have remained in the basic medium to make this possible. Whether or not this is true must await further study.

SUMMARY

Three purified members of the vitamin B complex were studied in respect to the nutrition of the ciliate protozoan, *C. striatum*, in bacteria-free cultures. Crystalline thiamin chloride (vitamin B₁) was proved to be a limiting factor for growth in media free from this substance. Heating a casein digest, tryptone, in the autoclave for one hour at 20 lb. pressure and pH 9.6 destroyed most of the thiamin. Vitamin B₁ was most effective in concentrations ranging from 1:10,000 to 1:10,000,000. Crystalline riboflavin and vitamin B₆ (concentrate) could not supplant thiamin in the nutrition of this ciliate.

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RATE OF GROWTH OF DAPHNIA LONGISPINA AS AFFECTED BY FISH-CONDITIONED WATER AND BY CERTAIN FISH EXTRACTS¹

(One figure)

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RESULTS of studies by Allee and associates have shown that, other things being equal and under the conditions of their experiments, isolated goldfish grew more rapidly if placed in water in which other goldfishes had lived than if in uncontaminated but otherwise similar water. Further investigations bear out the foregoing statement and extend the work to include assays of a protein extract of the surface of goldfishes (Allee, Oesting, and Hoskins, 1936).

The investigations reported here were the result of a bringing-together of the senior author's interest in the physiology of *Daphnia* with this work on the growth-promoting qualities of fish-conditioned waters and of extracts of fish origin. This afforded an opportunity to test the value of *Daphnia* as an assay organism for these growth-promoting substances and to ascertain the effect of heterotypic conditioning on the growth of *Daphnia*.

Kawajiri (1928) concluded from studies on population density of rainbow trout that the survival rate increased and the rate of growth decreased as the number of fry in a box increased. Adolph (1929) found that the maximum growth of amphibian larvae occurred with a single animal in the largest volume, although conditioned water could favor the growth of the individual. In 1932, Shaw working with the tropical fish *Platy-poecilus maculata* and with *Amblystoma* tadpoles found that heterotypic conditioning favored growth more than did homotypic conditioning. Woodruff (1914) showed that the excretory products of *Paramecia* were toxic to themselves, as was also true of hypotrichs. The excretory products of hypotrichs, however, stimulated the reproductive rate of *Paramecia*.

Although many of the factors involved are not yet entirely clear, heterotypic conditioning apparently favors growth more than does homotypic conditioning, and hence assays of reputed growth-promoting materials are more readily made across than within species boundaries.

Results to be reported in this paper include tests of fish-conditioned waters, a crude extract of the skin of goldfishes, and a refinement of the crude extract designated as alkaline extract, for their effects on the growth of *Daphnia*.

Extracts were prepared by placing goldfish (*Carassius auratus*) into 0.025 N NaOH for one hour; the resulting fluid which resembles a clear syrup was strained through heavy cheesecloth, and the volume divided into two equal parts. One portion was used with-

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out further treatment and designated as "crude extract." The other portion was treated with 0.5 N H_2SO_4 until a heavy precipitate was formed. The precipitate, which was washed thoroughly and dried with absolute ethyl alcohol and ether, was weighed, and its weight was assumed to be the equivalent of the extracted portion remaining in the crude state. This dry precipitate dissolved in 0.01 N NaOH constituted the "alkaline extract." The solutions of both extracts were autoclaved for 15 minutes at 15 lbs. pressure and stored in an icebox. Extract controls consisted of NaOH in aqueous solution added to the culture media of *Daphnia* so that the alkalinity of the control would be approximately the same as that of the test.

Several types of fish-conditioned waters were used, namely, conditioned lake water (C.L.W.) from Lake Michigan, conditioned tap water (C.T.W.), charcoal filtered, and conditioned synthetic pond water (C.S.P.W.) (Allee, Oesting, and Hoskins, 1936). These conditioned waters were prepared for use in experiments, concurrently in progress, dealing with their effects on assay goldfish and were thus prepared anew daily.

Female *D. longispina* from Banta's Line 1284 were used as the experimental animals. They afford certain advantages for investigations herein reported. Data for critical comparisons may be obtained from a large number of animals, and many experimental groups with adequate controls may be included in a single series. A still more important advantage which *Daphnia* afford arises from the fact that they reproduce by diploid parthenogenesis and as members of a pure line (clone) exclude the probability of a differential influence of any heritable factors for growth.

The *Daphnia* were reared individually in 100 cc. of manure-infusion medium (Banta, 1921) maintained at 25° C. and contained in a 6-ounce widemouthed bottle. A small outside fish tank containing several goldfish served as the source of water for manure-infusion media of Series III. The source of water for Series IV, V, and VI, except normal control of Series VI, which utilized water from the outside fish tank, was tap water filtered through activated charcoal and gravel. The animals were measured (body length exclusive of the caudal spine) with the aid of an ocular micrometer at the time of their release from the mother's brood chamber, and each brood was then distributed evenly among the various experimental and control groups. They were measured at the beginning of each instar up to and including the sixth and were transferred to fresh medium after each measurement. *Daphnia* do not increase in size except immediately following ecdysis, hence each measurement represents the summation of growth during any given instar.

The dilution for the extracts was 1 part fish extract to 400,000 parts culture medium. Media treated with fish-conditioned water were prepared by adding 20 cc. of conditioned water of known strength to 80 cc. of regular manure-infusion medium so that the coefficient of conditioning (Allee, Oesting, and Hoskins, 1936) was 25, except for one case in Series III where a set with a coefficient of 10 was also prepared. Control bottles, similarly, had 20 cc. of untreated lake, pond, or filtered tap water added to the 80 cc. of regular medium.

Table 1 presents a composite view of the results obtained. In Series III, the averaged first growth increment for the experimental groups was significantly³ larger than their controls, as is shown in the column of statistical probabilities.

³ Statistical significance was calculated in terms of P and is based on "Student's" method for finding statistical significance of relatively small numbers of paired observations. $P = 0.05$ is equivalent to three times the probable error; this value is taken as the extreme upper limit of statistical significance.

TABLE 1

INCREASE IN BODY LENGTH BY INCREMENTS OF *D. longispina* AS AFFECTED BY VARIOUS SUBSTANCES OF FISH ORIGIN

Treatment*	No. of Animals	Birth Length (Mm.)	First Incre- ment (Mm.)	Statistical Probabilities	Second Incre- ment	Statistical Probabilities	Third Incre- ment	Statistical Probabilities	Fourth Incre- ment	Statistical Probabilities	Total Body Length (Mm.)	Statistical Probabilities	Fifth Incre- ment	Statistical Probabilities	Total Body Length (Mm.)	Statistical Probabilities
Series III:																
Norm. cont.	13	.714	.179	.269	.237	.007	.237	.007	.227	.073	1.626	.199	.199	.001	1.825	.001
Cond. l.w. (to)	14	.711	.210	.252	.303	.000	.303	.000	.219	.150	1.695	.214	.214	.001	1.909	.001
Cont. l.w. (to)	14	.713	.179	.264	.252	.007	.252	.007	.252	.073	1.660	.198	.198	.001	1.858	.001
Cont. l.w.	13	.707	.211	.237	.204	.002	.204	.002	.223	.043	1.672	.265	.265	.002	1.937	.002
Cont. l.w.	13	.719	.183	.237	.238	.002	.238	.002	.223	.043	1.668	.198	.198	.002	1.866	.002
Alk. ext. I.	12	.716	.211	.258	.209	.001	.209	.001	.225	.012	1.700	.236	.236	.001	1.945	.001
Alk. ext. cont.	12	.706	.185	.273	.266	.000	.266	.000	.207	.033	1.637	.172	.172	.000	1.809	.000
Alk. ext. II.	12	.713	.204	.260	.325	.000	.325	.000	.226	.012	1.728	.260	.260	.001	1.988	.001
Crude ext. I.	13	.710	.210	.270	.310	.000	.310	.000	.259	.012	1.750	.236	.236	.001	1.995	.001
Crude ext. cont. I.	13	.725	.180	.256	.243	.000	.243	.000	.220	.012	1.624	.186	.186	.001	1.810	.001
Crude ext. II.	13	.717	.214	.267	.304	.000	.304	.000	.254	.012	1.756	.248	.248	.001	1.994	.001
Crude ext. cont. II.	12	.713	.179	.294	.235	.000	.235	.000	.221	.012	1.641	.181	.181	.001	1.843	.001
Series IV:																
Norm. cont.	20	.650	.172	.293	.277	.000	.277	.000	.319	.000	1.711	.053	.053	.000	1.764	.000
Cond. filt. t.w.	20	.650	.204	.263	.335	.000	.335	.000	.213	.000	1.665	.125	.125	.000	1.790	.000
Cont. filt. t.w.	20	.650	.173	.297	.276	.000	.276	.000	.238	.000	1.681	.065	.065	.000	1.746	.000
Alk. ext. I.	20	.649	.207	.270	.344	.000	.344	.000	.253	.000	1.723	.136	.136	.000	1.859	.000
Alk. ext. cont.	20	.647	.172	.297	.283	.000	.283	.000	.324	.000	1.703	.090	.090	.000	1.793	.000
Alk. ext. II.	20	.645	.202	.270	.338	.000	.338	.000	.255	.000	1.730	.141	.141	.000	1.871	.000
Cond. synth. p.w.	20	.643	.208	.264	.336	.000	.336	.000	.265	.000	1.647	.146	.146	.000	1.793	.000
Cont. synth. p.w.	19	.644	.179	.283	.285	.000	.285	.000	.306	.000	1.634	.072	.072	.000	1.706	.000
Crude ext. I.	20	.650	.212	.277	.354	.000	.354	.000	.255	.000	1.748	.191	.191	.000	1.939	.000
Crude ext. cont. I.	20	.651	.176	.297	.294	.000	.294	.000	.325	.000	1.743	.101	.101	.000	1.844	.000
Series V:																
Norm. cont.	15	.645	.176	.268	.247	.000	.247	.000	.340	.000	1.676	.295	.295	.000	1.971	.000
Cond. filt. t.w.	15	.645	.214	.253	.324	.000	.324	.000	.333	.000	1.779	.286	.286	.000	1.965	.000
Cont. filt. t.w.	15	.651	.171	.286	.235	.000	.235	.000	.331	.000	1.711	.252	.252	.000	1.906	.000
Alk. ext. I.	15	.638	.210	.234	.283	.000	.283	.000	.276	.000	1.641	.290	.290	.000	1.931	.000
Alk. ext. cont.	15	.635	.172	.297	.281	.000	.281	.000	.320	.000	1.662	.300	.300	.000	1.961	.000
Alk. ext. II.	15	.644	.208	.236	.305	.000	.305	.000	.303	.000	1.670	.273	.273	.000	1.949	.000
Cond. synth. p.w.	15	.639	.214	.252	.339	.000	.339	.000	.339	.000	1.800	.297	.297	.000	2.097	.000
Cont. synth. p.w.	15	.642	.173	.286	.289	.000	.289	.000	.303	.000	1.717	.252	.252	.000	1.906	.000
Crude ext. I.	15	.649	.210	.262	.341	.000	.341	.000	.326	.000	1.788	.308	.308	.000	2.096	.000
Crude ext. cont. I.	15	.650	.172	.293	.256	.000	.256	.000	.349	.000	1.720	.264	.264	.000	1.984	.000
Series VI:																
Norm. cont. (f.t.w.)	20	.678	.188	.265	.318	.000	.318	.000	.322	.000	1.771	.241	.241	.000	2.012	.000
Norm. cont. (p.w.)	20	.672	.193	.278	.334	.000	.334	.000	.305	.000	1.782	.282	.282	.000	2.064	.000
Cond. synth. p.w.	20	.673	.220	.248	.306	.000	.306	.000	.295	.000	1.796	.328	.328	.000	2.124	.000
Cont. synth. p.w.	20	.671	.186	.274	.317	.000	.317	.000	.341	.000	1.792	.241	.241	.000	2.033	.000
Alk. ext. III.	20	.668	.220	.246	.306	.000	.306	.000	.310	.000	1.804	.318	.318	.000	2.132	.000
Alk. ext. cont.	20	.677	.179	.283	.324	.000	.324	.000	.337	.000	1.790	.248	.248	.000	2.038	.000
Alk. ext. II.	20	.671	.190	.262	.324	.000	.324	.000	.334	.000	1.790	.240	.240	.000	2.030	.000

* Norm. = normal; cont. = conditioned; alk. = alkaline; ext. = extract; filt. t.w. = filtered tap water; synth. p.w. = synthetic pond water; p.w. = pond water.

The second growth increment presents a rather surprising picture. Most of the differences are insignificant, but the general trend is the opposite of that shown during the previous instar. During this instar, the controls showed a greater amount of growth as measured by increase in body length than did experimental groups. This point will be discussed in more detail later. During the third growth period there was a repetition of the trend shown during the first, namely, the experimental animals increased in body length significantly more than their respective controls. The fourth increment resembles the second, i.e., controls increased more in body length than did the experimental groups, although the differences are not statistically significant. A point of interest to the authors was to ascertain whether experimental groups, which had shown two increments (second and fourth) during which their increase in body length was less than their controls, were larger animals or whether they showed, in spite of their excessively rapid growth during two instars, no statistically significant difference from their respective controls in regard to body length. The average size (body length) of these animals is represented in the table as total body length, immediately following the fourth increment. As the data show, the experimental animals of Series III were, with the exception of those reared in conditioned lake waters, significantly greater in body length than their controls. However, when the various series are considered, the picture is not entirely clear since in Series IV, V, and VI, as shown in the table, the differences in total body length after four increments are not generally significant ones, although for the most part the experimental animals are the larger. Further experimentation will be necessary before this point is entirely clear.

Since, physiologically, the first five instars comprise a definite period in that the first brood of young is released at the end of the fifth instar, there was a particular reason to carry the animals through to the end of this interval. It is interesting to note, then, that the fifth growth increment (beginning of the sixth instar) was, in all the experimental groups, in line with the results of the first and third increments.

Series IV, V, and VI confirm to a great extent the results obtained for Series III. These added data clarify one point of importance, namely, for the second growth increment the control groups showed a growth increase which was in each case significantly greater than the increments for their respective test groups. This was not usually true for Series III. The same general alternation of a relatively large increment with a relatively smaller one continues to exist in the case of the test animals of the three latter series. However, even with these added data it is clear that many of the points need further confirmation and clarification.

Even though the results for the separate series bring out certain points of importance, the authors wished to have a composite picture of the effects produced by the several types of treatment. For this purpose the average values, already discussed, for the differences in growth increments between experimental groups and their comparable controls were assembled and averaged. This series of values for the mean of such sets of differences is represented in Figure 1 by the array of histograms for each group and instar. Each column by its height, then, indicates the degree of positive or negative difference when the base line is, in each case, the control appropriate to the experimental group. The degree of statistical significance or nonsignificance is indicated for each column in the graph. In the figure *B*—Alkaline extract includes all groups which were subjected to that type of treatment. In several cases, however, alkaline extracts were used which were old, and the primary purpose of their inclusion was to determine their possi-

ble value after aging. Accordingly, for C—Alkaline extract, the data based on the older preparations have been omitted because it was obvious that these extracts had lost, at least partially, their growth-promoting properties. The omitted groups include alkaline extract 1 and 2 of Series V and alkaline extract 2 of Series VI.

As shown in the figure, there is little difference between the averaged body lengths at birth. The increase in length of the first increment was significantly greater for the experimental groups. The results of the second increment are in agreement with those of each individual series, namely, the controls showed greater body-length increases than did the experimental groups. This difference was statistically significant except in the case of the crude extract (D).

The regularity of alternation between a relatively large increment one instar and a relatively smaller one the next for the experimental animals is clearly evident in the figure, although in the fourth instar a significant difference occurred only with conditioned water. The last group of histograms (black portion) shows that after four increments the experimental animals were actually larger, although the difference was significant in only one case, that of Alkaline extract—C. The others, however, approach significance, and results which would include a greater number of series might possibly demonstrate significance in each case.

The completely black-plus-white histograms in the last group show that when the first five summated increments of each experimental group were plotted against their respective controls, significance was shown in each case. However, this is hardly a valid comparison because it involves three increments (1, 3, and 5) during which increase in size was greater for the experimental groups and only two (2 and 4) increments in which the controls exhibited the greater increase. The results do however indicate that these materials increased total growth during the physiological interval between birth and the release of the first brood of young.

Any attempt to explain the alternation of growth increments evidenced in the results can be at best only tentative. One possible explanation has additional support from the considerable number of observations which the senior author has made on the life-history of *Daphnia*. It has been observed that an extra large number of offspring in a single brood is usually followed by a brood containing a smaller number. Also, critical observations have given the information that an instar of relatively short duration followed by a large growth increment usually results in a smaller increment for the next instar. Possibly these responses have been magnified to some extent by the present experimental treatments and indicate that a refractory period in the following instar is a necessary sequence to a large growth increment. In other words, during an instar which results in a large growth increment there might possibly be an exhaustion of, or interference with, the ability to exercise the growth potential due to accumulated products of metabolism, thus necessitating a period of recovery before the growth potential can again be exercised to its full degree. However, as stated above, this explanation is, although plausible, a hypothesis.

An analogous phenomenon of alternation of growth has been noted by Gause (1934, p. 36). He points out that "... a *Paramecium* which divides rapidly tends to give rise to daughter-cells which divide more slowly, and vice versa." Gause presents, however, no explanation of this effect.

Since *D. longispina* use bacteria as their chief source of food in the experiments it is possible that the conditioned waters and various extracts were effecting a more rapid rate of increase among the bacteria, thus making available a more abundant food supply

for the *Daphnia* and thereby making it possible for them to grow more rapidly. If such was the case, the foregoing experiments did not measure the effect of the various test materials on rate of growth of *Daphnia* but rather the effect of food supply on growth. However, the volume and concentration of the manure-infusion media used for both experimental and control groups was sufficient to supply, presumably, an overabundance of food to the animals at all times. The amount of available food is a very important factor in determining either directly or indirectly the brood size as well as rates of growth (Ingle, Wood, and Banta, 1937), and in the present experiments the numbers of young in the first broods (an average of about 13 per mother) released by the experimental and control animals were very similar to, as well as comparable with, the number ordinarily released by this species under normal laboratory conditions and at a temperature of 25° C. Thus it appears that the test substances were acting directly upon the *Daphnia*.

It might also be argued that the fish-conditioned waters and fish extracts, even though greatly diluted, might serve as an added source of food for the *Daphnia*. In this case, also, the overabundant food supply already present would tend to minimize the operation of this possible factor. In addition to this, it is doubtful whether *Daphnia* are capable of utilizing dissolved foods for their sustenance (Gellis and Clarke, 1935), although colloids are apparently necessary for their development; however, the presence of particulate food of ordinary culture media insures a more rapid development.

SUMMARY AND CONCLUSIONS

1. *D. longispina*, with few exceptions as noted in Table 1, showed a significantly greater growth increment for the first, third, and fifth instars when fish-conditioned water or extracts from the skin of fishes (*C. auratus*) were added to the manure-infusion medium.

2. The second and fourth growth increments of the test groups were smaller than their respective controls; this alternation suggests a refractory period as a necessary sequence to a large growth increment.

3. *Daphnia* of the test groups were, at the time of completion of four growth increments, larger animals (measured by body length), although in most cases not significantly so, but were at the end of five growth increments significantly larger animals in each case than their respective controls.

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AN ANALYSIS OF THE EFFECT OF NUMBERS UPON THE OXYGEN CONSUMPTION OF *CARASSIUS AURATUS*¹

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THE investigations reported here represent a continuation of research on an aspect of the mass physiology of animals—the effect of grouping on the activity of fishes (Shlaifer, 1938). Schuett (1934) found that an isolated goldfish, *Carassius auratus* Linné, was more active in an aquarium than were fishes in a group of four in the same volume of water. Escobar, Minahan, and Shaw (1936) confirmed these findings. Breder and Nigrelli (1938) also demonstrated the increased locomotor activity of *C. auratus* when isolated than when in a group of four. There remained, however, the conflicting reports of Schuett (1933) and Bowen (1932) as contrasted with the later work of Schuett (1934) concerning the relation between isolation and oxygen consumption in the goldfish. Schuett's later and more careful work indicated that there was no difference in the amount of oxygen used by a goldfish whether it was isolated or a member of a small group. Shlaifer (1938), measuring the activity of goldfishes indirectly through oxygen consumption and directly through observation, was able to resolve the conflict between studies on locomotor activity and studies on the use of oxygen. He found, among other things, that an isolated animal consumed more oxygen and had a higher rate of locomotor activity than did each fish in a group of two or of four.

The data in the present paper were collected in an attempt to analyze the factor or factors involved in the now well-established "group effect" in *C. auratus* and perhaps thereby to facilitate the analysis of the integrating mechanisms and behavior of fish aggregations in general.

MATERIALS AND METHODS

An extensive description of the general methods used may be found in a previous paper (Shlaifer, 1938). All experiments were performed in a room in Whitman Laboratory which has north windows only; the experiments were conducted between the hours of 9:00 A.M. and 12 noon. At the conclusion of a day's experiments, the fishes were placed in tap water, filtered free of chlorine and sediment, for a 21-hour period of acclimatization. The water was not changed until the conclusion of the experiments on the following day; this eliminated any disturbing effect of handling. Air, bubbled through the aquariums during acclimatization, maintained a normal oxygen tension. With one exception all the animals used were specimens of the common goldfish 5 cm. in length. The animals used for the experiments summarized in A of Table 1 were larger, measuring 8–10 cm. in length; these experiments were performed six months before those summarized in B of Table 1. The technique used throughout was the Rideal-Stewart modification of the Winkler method. The amount of oxygen used is a less direct indicator of activity than is the observation of locomotion. However, its measurement was deemed preferable for several reasons. In the first place, the observational method is not well adapted for sev-

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eral of the types of experiments performed—e.g., darkness tests. In the second place, there is good evidence (Shlaifer, 1938) that the measurement of oxygen consumption is free from many of the sources of error that are inherent in the observational technique and is, in general, more sensitive. Possibly it reflects in time such subtle reactions as the movement of the fins or changes in the rate of heart beat caused by stimuli received from other members of the group. Since the activity of individual fishes may vary to some extent and since the activity of any one animal may also vary relatively from day to day, it was necessary to perform a fairly large number of experiments with a large number of animals in order to minimize this source of variation. This was especially true for the oxygen-consumption tests which preclude the measurement of the activity of any single fish in a group; it is also true for experiments utilizing the observational method, since this is less sensitive. In the past investigators along these lines have sometimes made generalizations hardly warranted by the data, considering the known sources of variation. When the oxygen consumption of isolated animals was compared with that of animals in a group of four, equal sampling was effected by performing four times as many experiments on the isolated fishes. These same isolated animals were later used in the groups of four.

EFFECT OF DARKNESS

Among the vertebrates, vision is a particularly important factor in the integration of aggregations, schools, and flocks of fishes and birds. Welty (1934) found that goldfishes become conditioned to run a simple maze more rapidly when grouped than when isolated. The group seemed to stimulate learning behavior through various ways which were all visually integrated. The importance of vision in the schooling of fishes has been shown by Parr (1927) for *Pneumatophorus grex* (Mitchill), by Breder (1929) for *Jenkinsia stolifera* (Jordan and Gilbert), by Spooner (1931) for *Morone labrax* Linnaeus, by Bowen (1931) who found that the closely schooling catfish, *Ameiurus melas* (Rafinesque), failed to aggregate in the dark, and by Breder and Nigrelli (1935) for *Lepomis auritus* Linnaeus.

If vision is a dominant factor in the effect of grouping on the respiratory activity of goldfishes, this effect should disappear in darkness. The experiments described below explore this possibility.

Methods.—Control tests were performed with fishes placed in isolation and in groups of four in ordinary daylight, free from sunlight. At the conclusion of a day's experiments the water was changed, and the aquariums were placed in a dark box which was known to be light tight. The oxygen consumption of the fishes in this experimental condition was measured in a three-hour test period after 21 hours of acclimatization. The same animals were acclimatized and tested for the same period of time in daylight as in darkness. Those tested in isolation were subsequently placed in groups of four, while those in groups of four were later isolated. In the experiments otherwise performed in darkness, the aquariums were removed from the dark box just prior to the test period in order to pour on the seal of mineral oil and to obtain several samples of water for oxygen analysis. This sudden change in illumination may have been a source of artificial stimulation, but was, of course, the same for isolated and grouped animals. An attempt was made to minimize this condition by drawing the shades of all windows and placing a dark cloth over each aquarium until the samples were obtained. Animals that were not in use were kept in a large aquarium in ordinary daylight or under general illumination from ceiling lights when these were on at night.

Results.—Reference to Table 1 shows that in both sets of experiments, A and B, in daylight, the oxygen consumption of the isolated animals is significantly higher than that of fishes in a group of four. This amply confirms previous experiments covering this point (Shlaifer, 1938). In total darkness, on the other hand, there is no significant difference. Thus, it is seen that the "group effect" disappears in the absence of light. Examining the data further, we find that in both A and B the oxygen consumption of fishes in a group of four is not significantly higher in daylight than in darkness. Isolated animals, however, have a distinctly higher rate of oxygen consumption in the light. Thus, the

TABLE 1
EFFECT OF DARKNESS ON THE AMOUNT OF OXYGEN CONSUMED
PER FISH BY GROUPED AND ISOLATED GOLDFISHES

EXPERIMENTAL CONDITION	TEST PERIOD (HOURS)	I. ONE FISH			IV. FOUR FISHES		
		No. of Cases	Mean Cc. O ₂ Con- sumed per Fish		No. of Cases	Mean Cc. O ₂ Con- sumed per Fish	
			Daylight	Darkness		Daylight	Darkness
A*, §, †.....	3	32×1	7.44	6.10	8×4	6.01	5.70
B§, †, ‡.....	3	32×1	3.82	2.90	8×4	2.72	2.60

* = 8-10-cm. fish.

† = In 7.5-liter aquarium.

‡ = In 6 liters of water.

§ = 5-cm. fish.

TABLE 1a
STATISTICAL ANALYSIS OF TABLE 1 SHOWN IN *P*-VALUES*

Experimental Condition	I vs. IV—Light	I vs. IV—Dark	I—Light vs. I—Dark	IV—Light vs. IV—Dark
A.....	0.026	0.590	0.0002	0.380
B.....	0.0048	0.406	0.0000	0.264

* Upper limit of statistical significance is set at 0.05. This is three times the probable error. *P* = 0.01 indicates good significance, while a value of 0.100 or more indicates little significance ("Student," 1925).

group effect appears to be eliminated not through increased use of oxygen by grouped fishes in the dark but by the decreased consumption of the isolated animals in that state.

These data indicate the importance of vision as a factor in the lessened activity of grouped goldfishes..

EFFECT OF BLINDNESS

If the group effect is lost in total darkness, indicating a role of vision, it follows that the same results should obtain with blinded fishes. Parr (1927) found that blinded individuals did not mill or exhibit schooling reactions. Bowen (1931) found that blinded catfishes did not aggregate.

Methods.—A group of individuals from set B of the darkness tests were blinded by completely removing both eyes. Though more drastic, this method seemed to be prefer-

able to covering the eyes with opaque materials which might wear off. Several animals did not survive the operation, and only those were used which, except for their blindness, exhibited normal behavior, at least as judged by the observer. The fishes that died did so in a few days; the survivors were observed for several weeks before they were used. These animals lived for months after the experiments were completed, indicating excellent recovery from the operation. The blinded fishes were placed alternately in isolation and in groups of four in daylight, and their oxygen consumption was measured in a test period each day. Removal of their eyes does not permit the use of the experimental animals as control animals every other day as was possible in the tests in darkness. However, it should be borne in mind that before being blinded, these animals had been used in the preceding series of experiments and, when in ordinary daylight, showed a definite group effect.

Results.—The data in Table 2 indicate that when fishes are blinded, there is no significant difference in the oxygen consumption of grouped and isolated animals. When com-

TABLE 2
EFFECT OF BLINDNESS ON THE AMOUNT OF OXYGEN CONSUMED
BY GROUPED AND ISOLATED GOLDFISHES*

ISOLATED		GROUP OF FOUR		MEAN DIFFERENCE	P
No. of Cases	Mean Cc. of O ₂ Consumed per Fish	No. of Cases	Mean Cc. of O ₂ Consumed per Fish		
28×1	2 20	7×4	2 07	0 13	0 7186

* Test period, 3 hours; volume, 6 liters of water in a 7.5-liter aquarium.

pared with the fishes placed in darkness in B of Table 1, the blinded animals, themselves selected from set B, are seen to have a lower rate of oxygen consumption in both the isolated and the grouped states. This is partially a result of the fact that the surviving blinded animals were somewhat smaller than those in the original group. Thus, the elimination of the action of vision through blinding also eliminates the group effect.

VISUAL CONTACT

Though the darkness and blindness experiments show that the sense of vision is important, they do not rule out the possibility of other sense organs coming into play in the group behavior of the goldfish in an important though perhaps subtle manner. It is possible that vibrations of tails of fishes set up pressure disturbances, detected perhaps by the lateral-line sense organs, which influence the activity of grouped animals. Thus, in the strongly thigmotactic *A. melas* Bowen (1931) demonstrated such responses and touch as well in the aggregating behavior of this species. Even with these bullheads vision was found to be essential for aggregations since blinded fishes and normal ones kept in the dark did not aggregate.

The experiments reported below were designed to eliminate all factors but vision. If isolated goldfishes manifest a group effect when in contact through vision alone with

other goldfishes, it may be concluded that vision is a very important factor in their normal group behavior.

Methods.—A 3.5-liter aquarium with dimensions of 17.5 cm. by 13 cm. and 20 cm. deep was placed in a 15-liter aquarium whose dimensions were 30 cm. by 27 cm. and 24 cm. deep. The walls of both aquariums were of transparent glass. The smaller aquarium was filled with 2.5 liters of water which gave a depth of 13 cm. at the mark. The larger aquarium was filled with enough water, about 6 liters, so that, when the smaller one was placed in it, the two water levels coincided. On each of the four vertical sides there were 5 cm. of space between the inner surface of the large aquarium and the outer surface of the small aquarium. One goldfish was placed in the smaller aquarium. In the control tests there were no animals in the outer aquarium, while in the experimental condition there were three goldfishes present. The animals used in all experiments were rather small—5 cm. in length; this permitted sufficient free movement in either aquarium. The usual oxygen seal of mineral oil was placed on the water surface of

TABLE 3
EFFECT OF VISUAL CONTACT WITH OTHERS OF THE SAME SPECIES ON THE
AMOUNT OF OXYGEN CONSUMED BY ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	P
No. of Cases	Mean Cc. of O ₂ Consumed	No. of Cases	Mean Cc. of O ₂ Consumed		
16	1.99	16	1.32	0.67	0.0002

* Test period, 3 hours; volume, 2.5 liters of water in a 3.5-liter aquarium.

† Isolated fish in no visual contact with other fishes.

‡ Isolated fish in contact through vision alone with three other fishes.

the small aquarium only. The oxygen consumption of the isolated animal in the small aquarium was measured each day. The isolated fish was alternated daily between complete isolation and contact through vision alone with three other animals of the same species. Several fishes were tested in the course of the experiments. One might be inclined to doubt whether the optical powers of the goldfish are sufficiently acute to enable the isolated animal in the foregoing setup to respond to its fellows in the outer aquarium. However, Welty (1934) in the course of his experiments on learning in the goldfish showed that these animals learn to swim a simple maze more rapidly after having seen, through transparent glass, other fishes perform in it.

Results.—Table 3 shows a significant decrease in the oxygen consumption of the isolated fish in the inner aquarium when in visual contact with three others of its kind.

Since all other stimuli but those due to vision have been eliminated, this experiment indicates that visual response to other fishes is a dominant factor responsible for the group effect in goldfishes which is indicated by the decreased rate of activity and oxygen consumption in a group of four as contrasted with similar data for isolated animals.

EFFECT OF MIRROR IMAGES

This experiment was designed to investigate further the role of visual stimuli in aggregations of *C. auratus*. Also, it furnishes an interesting sidelight on general fish behavior. Vol. XII, No. 4, OCTOBER, 1939]

havior. Spooner (1931) found that the bass—*Morone labrax*—a vigorous fish with a rather strong tendency to school, gave definite reactions to a mirror. An isolated fish in contact with a mirror would often lie motionless against it for extended periods of time. When Spooner (1931) used mirror surfaces that were broken up by running elastic bands across the surface of the mirror at definite intervals, the fishes reacted distinctly to these mirrors if the bands were three-quarters of an inch apart; at smaller intervals there were either weak reactions or none at all.

Methods.—7.5-liter aquariums containing 6 liters of water were used. The aquariums used for the control tests had four transparent vertical glass sides; those used for the experimental condition were mirrored on two contiguous vertical sides which formed a right angle. The remaining two vertical sides of the mirrored aquariums, also forming a right angle, were constructed of transparent glass. Isolated animals were alternated each day between the plain and mirrored aquariums, and their oxygen consumption was measured in a 3-hour test period.

TABLE 4
EFFECT OF MIRROR IMAGES ON THE AMOUNT OF OXYGEN CONSUMED
BY ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	P
No. of Cases	Mean Cc. of O ₂ Consumed	No. of Cases	Mean Cc. of O ₂ Consumed		
22	2.92	22	2.09	0.83	0.0000

* Test period, 3 hours; volume, 6 liters of water in a 7.5-liter aquarium.

† Isolated fish in a clear-sided aquarium.

‡ Isolated fish in an aquarium mirrored on two adjacent sides.

Results.—The data in Table 4 show a significant fall in the oxygen consumption of the isolated fish when in the mirrored aquarium. All factors but vision have again been eliminated. The animal in the mirrored aquarium tended to remain near and parallel to the mirrored surfaces, apparently reacting as though it was lining up with another fish in a group of two.

The experiments described above leave little doubt as to the important role played by sight in these group reactions and make unnecessary the analysis of other factors at this time. Granted that this sense is so important in the responses tested, how does it act? The fishes may be reacting to (a) color, (b) form, (c) type of movement, (d) amount of movement, or (e) some combination of these factors.

REACTION TO COLOR

The response of fishes to colors is a field which still requires investigation. In a comprehensive review Warner (1931) criticized the lack of control of the intensity factor and was of the opinion that most of the experimental work would bear repeating. White (1919, 1927) demonstrated that mud minnows and sticklebacks can discriminate wavelengths and not merely intensities of light. Noble and Curtis (1935) found that when the female cichlid, *Hemichromis bimaculatus*, was given a choice between a male with ery-

throphores expanded by yohambine and a paler control, she usually selected the former. Brown (1937) demonstrated the reaction of the largemouthed black bass, *Aplites salmoides*, to colors.

Methods.—The aquarium setup was the same as that described for the visual-contact experiments. In the control setup three normal orange-colored goldfishes were placed in the large aquarium. In the experimental condition, these animals were replaced by three goldfishes of the same size and shape and of a dark gray or black color, mixed with a slight trace of orange. In general, at least to the human eye, the experimental animals formed a distinct contrast to those used in the control tests. Isolated normal orange-colored goldfishes in the smaller aquariums were alternately placed in visual contact with the orange and with the dark animals, while their oxygen consumption was measured as usual.

Obviously, these experiments were not designed to test the reactions of *Carassius* to color as such. Rather, they are naturalistic in nature and do explore the possibility that orange goldfishes respond differentially to differently colored members of their species.

TABLE 5
EFFECT OF VISUAL CONTACT WITH ORANGE OR WITH DARK INDIVIDUALS
ON THE OXYGEN CONSUMPTION OF ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	P
No. of Cases	Mean Cc of O ₂ Consumed	No. of Cases	Mean Cc. of O ₂ Consumed		
24	1 71	24	1 61	0 10	0 363

* Test period, 3 hours; volume, 2.5 liters of water in a 3.5-liter aquarium.

† Isolated fish in visual contact with three normal orange goldfishes.

‡ Isolated fish in visual contact with three darkly colored goldfishes.

Results.—Reference to Table 5 shows that there is no significant difference in the rate of oxygen consumption of an isolated fish when it is in visual contact with a goldfish of a distinctly darker color than the normal orange type. Thus, the major visual response of fish to fish is apparently not in terms of color. It may be that the technique and experimental setup were not sensitive enough, but the general indications are that other factors are involved.

REACTION TO BODY FORM AND MOVEMENT

The factors that remain to be analyzed are the reactions to body form and to movement. Spooner (1931) found that a fish will be attracted to and will lie alongside a dead fish supported by glass in the position of a resting live one. Thus it is seen that a fish may be attracted by the form of another one that is completely devoid of movement. Apparently, the form in that case had to be a good replica of the living animal, for, when rough models of fishes were tried, no attraction was obtained.

The following experiment was designed to test the factors of form and movement simultaneously.

Methods.—The aquarium setup was again the same as that described for the visual-contact tests. Normal orange goldfishes were killed and injected with formaldehyde and

were mounted on sharply pointed glass rods attached at the base to hidden wire loops for support. These mounted animals were placed in the outer aquarium in the normal swimming position close to and parallel to the sides of the smaller aquarium. The fishes in both aquariums in that type of setup usually swim in a horizontal plane about 4 cm. above the bottom. Accordingly, the mounted animals were also placed at that level. No odor of formaldehyde, which had been injected into the dead animals, could be detected when they were in position in the aquarium. Three mounted fishes were placed in the larger aquarium in the experimental setup, two of them, nose to tail, next to one long side of the small aquarium; the third was placed in the opposite passage way and pointed in the same direction as the other two. When it was thought that the color of the mounted specimens was not quite normal, they were replaced by newly killed and mounted animals. The hollow transparent glass rods on which the fishes were mounted probably eliminated any possibility that the animal in the inner aquarium would respond to the mounting material as well as to the mounted fish. The isolated test animals in the inner

TABLE 6

EFFECT OF VISUAL CONTACT WITH NORMAL OR WITH MOUNTED INDIVIDUALS ON THE OXYGEN CONSUMPTION OF ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	P
No. of Cases	Mean Cc. of O ₂ Consumed	No. of Cases	Mean Cc. of O ₂ Consumed		
20	1.95	20	1.46	0.49	0.0001

* Test period, 3 hours; volume, 2.5 liters of water in a 3.5-liter aquarium.

† Isolated fish in visual contact with three normal goldfishes.

‡ Isolated fish in visual contact with three dead and mounted goldfishes.

aquariums were alternately placed in visual contact with the control setup of three normal orange goldfishes and with the experimental condition of the three killed and mounted specimens; their oxygen consumption was measured as usual in a 3-hour test period.

Results.—The data in Table 6 show that the oxygen consumption of the isolated fish is significantly lower when that animal is in visual contact with immobilized fishes. The interesting implications of this experiment are too lengthy to be treated here and are reserved for the following section.

DISCUSSION

The effect of grouping on the oxygen consumption and locomotor activity of fishes is an aspect of animal aggregations. Accordingly, as a somewhat minor result of these experiments, it is possible to suggest the place occupied by *C. auratus* in a system of classification of such aggregations. This becomes more interesting in connection with the fact that "... no hard and fast line can be drawn between well-integrated social organizations and loosely integrated aggregations which are usually regarded as being definitely non-social" (Allee, 1931, p. 14), since the goldfish is not a closely schooling species. Presumably, therefore, it occupies a low position even in the more inclusive estimates of the extent of sociality such as those given by Wheeler (1928) and Allee (1938).

In the system of classification of animal aggregations given by Allee (1931, p. 35, 36), the aggregating behavior of the goldfish might conceivably fall into the two major divisions given for individuals which are not organically connected: (1) aggregations primarily due to reactions to the environment and (2) aggregations primarily due to reactions to other organisms.

Theoretically, goldfish aggregations that fall into the first category may occur in nature. Thus, the animals may be attracted tropistically to some particular region of a pond that at a particular time presented optimal chemical or physical conditions. This type of aggregation is much less likely even in large aquariums or tanks in the laboratory; in smaller aquariums under controlled experimental conditions, it may practically be eliminated from consideration. For example, it is observed that goldfishes in a group of four may tend to occupy, more or less, a certain region in an aquarium, the region occupied being changed from time to time. When the animals are blinded, they are distributed at random indicating that the reaction was a result of integration with other individuals rather than caused by some differential factor in the environment.

Aggregations of goldfishes in nature probably fall much of the time in class 2; in laboratory aquariums, properly protected from any changes in the uniformity of the environment such as sunlight, proximity to heat, etc., category 2 would be the sole possibility.

In addition to the foregoing subdivisions of aggregations based on the method of integration, Allee (1931, p. 36) lists the following subdivisions based on the degree of integration:

1. Relatively slightly integrated groups in which the primary (individual) reactions predominate and whose survival value is apparent only after experimentation.
2. Moderately well-integrated groups in which the secondary (group) reactions predominate, although primary reactions are still strongly in evidence, and whose survival value is more obvious.
3. Highly integrated groups in which the primary reactions are decidedly in the minority and the social value is strongly in evidence.

Aggregations of *C. auratus* fall nearer to class 1 than to class 2. The closely schooling mackerel would fall definitely into class 2.

Shlaifer (1938) found that a goldfish has a higher rate of oxygen consumption and locomotor activity when isolated than when it is a member of a freely moving group. The experimental data presented here confirm these earlier results and extend them to demonstrate the importance of visual stimuli in decreasing the activity of grouped goldfishes. Visual contact of an isolated animal with freely swimming goldfishes or even with a mirror self-image retards activity. This is found to be independent of the color. The data listed in Table 6 also show that the activity of the isolated animal is greater if the fishes in the visual field are moving than if they are immobile. Therefore, the isolated goldfish in visual contact with the group of immobilized animals is affected in its behavior even more than if the fishes in its visual field are active. Under these conditions the isolated animal can be reacting to form and to color only. Since color, within the limits tested, is not needed to produce the usual group effect, it appears that, under the conditions tested, the isolated fish is reacting to form alone. Apparently, it is enough that a goldfish see its own mirror image or an immobilized goldfish in order to undergo the quieting effect of the group. The extent to which this form can be altered from that of goldfishes and still evoke a similar effect remains to be discovered by direct experimentation.

It was shown in the darkness experiments that the group effect is eliminated not

through an increase in oxygen consumption by the grouped fishes in the dark but by decreased consumption by isolated animals in that state. If we assume that darkness tends to act as a quieting factor for goldfishes, an explanation may be offered for the observed results as follows: The isolated animal, subjected to no stimuli from other fishes, is affected only by the change in illumination, and its activity is diminished; in the grouped animals, the quieting effect of darkness is equalized by the loss of visual inhibition of activity and, perhaps, by random contacts.

The dominance of vision as the factor that inhibits activity in groups of goldfishes can be further confirmed by comparing certain data in Tables 1 and 3. These experiments were selected for comparison because they are the only ones whose general conditions are similar enough for that purpose. Even these experiments were performed under setups that were sufficiently different to make impossible a perfect comparison. Thus, the oxygen consumption of the fishes in Table 3 is lower, regardless of the condition of grouping, than is that of the animals in B of Table 1; this is probably due to the distinctly lower volume of water available to the animals in the smaller aquarium used for the experiments summarized in Table 3. (A of Table 1 is not used in the comparison because the individuals in those experiments were considerably larger than those used later.) It is seen in B of Table 1 that the oxygen consumption of an isolated fish in daylight is decreased by 29 per cent when it is subsequently used as a member of a group of four. Reference to Table 3 shows that, when an isolated fish is made a member of a group of four through visual contact alone, its oxygen consumption is reduced by a similar amount—33 per cent. Thus, visual response to other fishes, taken alone, may produce a group effect of the same order of magnitude as does that shown when an individual is an actual member of a group of four.

Of the four factors involved in visual response—namely, color, form, type of movement, and amount of movement—only form has been found to act as an inhibitor of activity. The data in Table 6 indicate that inhibition of activity of an isolated goldfish is greater when it responds through sight to form *minus* movement than it is when the animal responds to form *plus* movement. This may mean that movement as such is a stimulator of activity. The role of the type of movement in aggregations of fishes is a much more subtle affair and may be investigated through the use of heterotypic groups.

Experiments on fishes in laboratory aquariums suffer from the criticism of being divorced from conditions as they occur in nature. However, the obvious necessity for the careful control of variables greatly mitigates this criticism. Apropos of this consideration, the experiments described in this paper may be listed in the following increasing degree of artificiality: (a) experiments with normal fishes in darkness, (b) experiments with normal fishes in daylight, (c) experiments with blinded fishes, (d) effect of mirror images, and (e) contact through vision alone with (1) normal fishes and (2) mounted fishes.

Many reactions of animals tested experimentally may never occur, as far as we know, in a natural environment. Nevertheless, it is important to know not only the normal behavior that usually is manifest but also the behavior potentialities of an organism. Very often it is the analysis of behavior under unnatural conditions which are carefully controlled that facilitates the explanation of normal behavior. Also, many factors in the behavior complex of an organism are so subtle as to necessitate artificial methods of analysis if they are to be discovered at all.

As discussed above, it is apparently the visual perception of body form that inhibits

the activity of grouped goldfishes. Granted that this is so, it is difficult to offer an explanation for this pattern of behavior. Breder and Nigrelli (1935) state that a schooling fish primarily holds a position of maximum "comfort" by fixing its vision on some object, either a stationary one or a moving one; this difference determines whether the fish maintains a stationary position or swims forward. If this also holds true for the goldfish, which is not a strongly schooling animal, it may be that the increased activity of the isolated animal, which cannot find an object on which to fix its vision, is a result of a state of "psychic unrest," a state which may well defy direct experimental exploration. Thus, as in most aspects of animal behavior, the ultimate analysis of the *modus operandi* remains as elusive as it is interesting.

One may speculate as to whether the reaction of a goldfish to the body form of others in a group is a response to the particular type of body form characteristic of the species or to body form of fishes in general. Escobar, Minahan, and Shaw (1936) worked with a heterotypic group of four fishes; the species used were *C. auratus*, *Gambusia affinis*, *Oryzias latipes*, and *Macropodus verdi-auratus*. The group effect found here for the goldfish was the opposite of that found for it in homotypic groups of four. The activity of the goldfish in the heterotypic group exceeded even that in an isolated condition. More extensive work with heterotypic groups in observing locomotor activity and measuring oxygen consumption and utilizing, perhaps, some of the experimental setups described above may yield fruitful and interesting results which would have significance for the general problem of the integration of animal aggregations. It is quite probable that any group effect that might be found in such heterotypic groups would be produced by a combination of factors distinctly more complex than is to be found in the homotypic groups used so far. Thus, the color, form, type of movement, and amount of movement might vary considerably when several species are used. The activity of a goldfish in heterotypic groups of three species might in some cases be found to be a resultant of two opposing tendencies caused by the other two species in the group. Perception of body form might stimulate activity in heterotypic groups, or its inhibitory action might be masked by some more dominant responses to type and amount of movement. It would be necessary, of course, to employ suitable techniques to isolate, as far as possible, the variables in this complex. Any conclusions drawn from the behavior of fishes in heterotypic groups would have to be based on a considerably larger group of data than is necessary for homotypic groups because of the variables involved. However, the implications, especially at the relatively low state of integration that is involved in such aggregations, appear to justify the work that would be necessary. Such an analysis is the more important because heterotypic aggregations have ecological implications of far-reaching significance in the study of animal communities in nature. Experiments along these lines are now under way in our laboratory.

Parr (1937) makes the interesting point that a fish with a rather rigid body—the mackerel, for instance—displays definite schooling behavior visually integrated, though it is incapable of self-perception. Therefore, Parr (1937) states: "... the reaction of the mackerel to the perception of others of its kind cannot be in terms of a response to individuals recognized to be similar to itself, but must be based upon an automatic association mechanism of a different order in which the perception of another fish similar to itself fits as a key fits into a lock." If this is true for the mackerel, it also holds for *C. auratus*. From this point of view, the proposed analysis of heterotypic aggregations of fishes presents new and interesting problems.

SUMMARY

1. The effect of numbers upon the oxygen consumption of grouped and isolated goldfishes, manifested by a decreased rate of oxygen use in the group, is lost when the animals are placed in the dark or are blinded.
2. The oxygen consumption of isolated goldfishes is decreased when they are placed in contact through vision alone with others of the same species, with their own mirror images, or with recently killed and mounted forms.
3. It is evident that vision is the major sense involved in this aspect of mass physiology.
4. Inhibition of activity in grouped animals is probably due to the perception of the form rather than the color or movement of the fishes comprising the group.

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A PERFUSING SOLUTION FOR THE CRAYFISH HEART AND THE EFFECTS OF ITS CONSTITUENT IONS ON THE HEART¹

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A SATISFACTORY solution for nerves of the crayfish, *Cambarus clarkii*, was developed by van Harreveld (1936) based upon chemical analyses of blood. Since its composition varied considerably from solutions reported by others to be satisfactory for crayfish, especially with respect to calcium, it seemed desirable to test it on the crayfish heart, to compare it with those other solutions, and to study the effects of constituent ions on the heart.

TABLE 1
COMPOSITION AND CATION RATIOS OF SOME OF THE SOLUTIONS
USED ON THE CRAYFISH HEART

SOLUTION	TOTAL MOLAR- ITY	NaCl MOLAR	KCl MOLAR	CaCl ₂ MOLAR	MgCl ₂ MOLAR	NaHCO ₃ MOLAR	CATION RATIOS WHEN Na = 100*		
							K	Ca	Mg
Crayfish blood (van Harreveld) . .	Δ° C. = 0.85		0.0051	0.0137	0.0028				
van Harreveld	0.2288	0.2051	0.0054	0.0135	0.0026	0.0022	2.63	6.58	1.27
Lindeman	0.1768	0.1700	0.00375	0.00216			2.19	1.26	
C	0.1062	0.1880	0.00402	0.00720			2.14	3.83	
Hoffmann	0.228	0.2200	0.004	0.002		0.002	1.82	0.91	

* Omitting the Na in NaHCO₃.

The composition of van Harreveld's solution is given in Table 1. The pH varied from 7.2 to 7.8, and the depression of the freezing point was -0.86° C. which agrees well with the depression for crayfish blood (-0.85° C.; van Harreveld, 1936). This solution was first compared with the one reported by Lindeman (1929); then with a solution having intermediate amounts of sodium and calcium chlorides (solution C); with double Ringer's solution, which Hoffmann reported he used, although the compositions given are incorrect (Hoffmann, 1912-13); with isotonic single salt and glucose solutions, and with thirty-one other salt mixtures, the ionic ratios of which covered many significant possibilities.

¹ Nearly all the experiments herein reported were done at the Kerckhoff Laboratories in the fall and winter of 1937. To Drs. T. H. Morgan, C. A. G. Wiersma, and to other members of the staff the senior author expresses sincere thanks for the privileges and courtesies extended to him.

Since the extensive report of Plateau (1880) on the heart of decapods, there have been published many accounts of attempts to perfuse the heart of crustacea and of a few other invertebrates in order to compare the cardiac behavior of vertebrates with that of invertebrates. The work of Carlson (1905, 1906, 1906-7) on *Limulus* is perhaps the best known. A great variety of perfusing solutions has been used, and, as might be predicted, conflicting and equivocal results have been obtained, so that the question of how the invertebrate heart differs from the typical vertebrate heart is still largely unanswered. One probable reason for confusion has been the frequent use of unbalanced and improperly antagonized perfusing solutions. In the experiments reported here it has become clear that for the crayfish heart a favorable perfusing solution must correspond, in its inorganic composition at least, to the crayfish blood. It is likely that the same thing will be found true of other invertebrates. A critical review of the literature will, therefore, be postponed until proper solutions have been developed for representative species.

METHODS

Crayfish of both sexes collected in the vicinity of Pasadena and varying in body length from 6 to 10 cm. were prepared for perfusion of the heart as follows. The larger part of the carapace between the eyes and the cervical grooves was removed. The stomach, "liver," and reproductive glands were then removed, with the animal held vertically, head downwards.

Alimentary or liver juices were found to be highly injurious if they came in contact with the heart. The cavity was thoroughly washed with the physiological solution, and the ganglionic nerves were severed by cutting along each side of the ganglionic chain directly under the pericardial region. The anterior section of the pericardium was then cut away, thus exposing the pericardial cavity. A small spring pinch clamp 8 mm. long, made of rustless steel wire, was fastened to the midanterior margin of the heart. The animal was securely fastened in a vertical position head upwards. A thread attached the pinch clamp to a heart lever using a fine human hair as a marker on a lightly smoked drum. The tip of a glass tube with bore of about 1.5 mm. and a funnel-shaped opening at the upper end, was fastened about halfway down in the pericardial cavity at one side of the heart. The experimental solution flowed into that tube at a constant rate (6 ± 1.0 ml./min.) and at room temperature ($22^\circ \pm 2.0^\circ \text{C.}$), filling the pericardial cavity and overflowing around the top of the latter. This method of perfusion was found to be far preferable to methods in which the crayfish is held in a horizontal position. Different perfusing solutions at the same rate of flow could quickly be substituted for van Harreveld's solution and compared with it. Clock time and frequency of beat, measured by a stop watch, were recorded on the smoked paper along with the heart record.

RESULTS

During the first few minutes of perfusion the frequency, tone, and amplitude of the heart beat varied considerably, but by the end of 15 minutes they usually became relatively constant. Of eighty-two animals used for perfusion by van Harreveld's solution, the normal rate at the end of 15 minutes varied from 62 to 118 beats per minute with an average of about 93. The heart beat of seven intact animals observed through Cellophane windows, between 20° and 23°C. , varied from 75 to 136, with an average of 116 per minute. In a large percentage of the preparations, the beat was extremely regular, and the amplitude of successive beats was constant. A few animals showed constant but

different amplitudes of alternate beats or rhythmic beats of more than two amplitudes or a continuous irregularity of rate and amplitude. Such hearts rarely ever became typical afterward.

Hearts perfused only with van Harreveld's solution continued normal beating for many hours (usually from 8 to 10 hours; occasionally from 9 to 14 hours, and a few from 18 to 20 hours). With occasional stimulation by lentin (carbamylocholine chloride) two hearts continued beating for 24 and 29 hours. There is little doubt that the other tissues of the animal were dead within 3 hours. It is believed that injury to the heart by the operation and by the pinch clamp is the most common cause of failure of hearts perfused only by that solution, if failure occurs before 5 hours. Since the solution contains no nutritive materials, no hemocyanin, and none of the other organic constituents of crayfish blood, it is not surprising that the heart stops eventually. Such arrest occurs in diastole and is characterized during the later hours by slowly decreasing frequency and amplitude without any significant change in tone.

Fifteen hearts were perfused only with Lindeman's solution, and in each case the frequency exceeded that found for van Harreveld's by from 10 to 50 per cent. Beating continued for much shorter periods of time (usually less than 2 hours; occasionally from 2 to 3 hours). Arrest was characterized by slight increase in tone, as well as by decreased frequency and amplitude. If hearts were first perfused by van Harreveld's solution and then changed to Lindeman's, marked increase in frequency and amplitude always occurred, followed by decreases in each, up to arrest in tonic diastole or one-half systole. If the solution was replaced by van Harreveld's before the frequency had decreased too much, the heart quickly recovered its normal characteristics. Obviously Lindeman's solution is much less satisfactory for the heart than van Harreveld's. The former contains no magnesium, is not buffered by bicarbonate, has a molarity 23 per cent less than the latter, and contains only one-fifth the latter's ratio of calcium ions to sodium ions. Results to be described later show that the absence of magnesium is relatively unimportant and that the lack of buffer action and the lower osmotic pressure are minor faults. The chief factor, therefore, in rendering Lindeman's solution unsatisfactory is the incorrect calcium content.

Solution C, with intermediate amounts of sodium and calcium and an intermediate molarity, also caused increased frequency and amplitude as compared with van Harreveld's, but to a lesser degree than Lindeman's. Furthermore, there was considerable adaptation, in that the frequency and amplitude returned to normal in some cases, and beating continued for from 2 to 4 hours. Double frog Ringer's solution (or Hoffmann solution, cf. Table 1), with the same molarity as van Harreveld's but with smaller amounts of potassium and calcium than Lindeman's, was found to be very unsatisfactory, causing marked increase in frequency and amplitude at first, followed by rapid decrease up to arrest in systole within 30 minutes. Varying the pH of van Harreveld's solution from 6.4 to 8.4 caused no significant changes in the frequency, tone, or amplitude of the heart beat. Below or above those limits changes in tone and amplitude usually occurred, although a detailed study of such changes was not made.

Contradictory results were often obtained during the early experiments, until it was discovered that the heart's behavior in the solutions might depend upon previous treatment. For example, if a beating heart which had been perfused with double Ringer's solution for a certain period was returned to van Harreveld's solution, temporary arrest in diastole might occur, although recovery to normal would usually follow within the

next hour. Subsequently, therefore, all results were checked on fresh hearts treated previously only with van Harreveld's solution.

To determine the relative roles of each cation and the best mixture of them, various significant combinations, the majority of which are displayed in Table 2, were tested. In all of the solutions the pH was practically the same owing to the buffering action of

TABLE 2
IONIC RATIOS, MOLARITIES, AND CHARACTERISTICS OF
SOLUTIONS USED ON THE CRAYFISH HEART

Solution	NaCl	KCl	CaCl ₂	MgCl ₂	NaHCO ₃	Total M	Characteristics
van Harreveld's....	0.205	0.0054	0.0135	0.0026	0.0022	0.2288	"normal"
1.....	0.217	0.0057	0.0026	0.0022	0.2275	No Ca
2.....	0.210	0.0138	0.0027	0.0022	0.2287	No K
3.....	0.207	0.0054	0.0136	0.0022	0.2282	No Mg
4.....	0.208	0.0046	0.0114	0.0026	0.0022	0.2288	-15% K
5.....	0.204	0.0063	0.0134	0.0026	0.0022	0.2285	+17% K
7.....	0.201	0.0098	0.0132	0.0025	0.0022	0.2287	+84% K
8.....	0.195	0.0051	0.0238	0.0025	0.0022	0.2286	+82% Ca
9.....	0.191	0.0093	0.0234	0.0024	0.0022	0.2283	+84% K, +74% Ca
10.....	0.188	0.0122	0.0232	0.0024	0.0022	0.2280	+125% K, +74% Ca
11.....	0.206	0.0054	0.0125	0.0026	0.0022	0.2287	-7% Ca
12.....	0.203	0.0054	0.0147	0.0025	0.0022	0.2278	+9% Ca
13.....	0.188	0.0100	0.0260	0.0024	0.0022	0.2286	+85% K, +92% Ca
14.....	0.214	0.0027	0.0068	0.0027	0.0022	0.2284	-50% K, -50% Ca
17.....	0.202	0.0053	0.0160	0.0026	0.0022	0.2281	+18% Ca
18.....	0.205	0.0054	0.0128	0.0026	0.0022	0.2280	-5% Ca
19.....	0.1025	0.0027	0.0067	0.0013	0.0022	0.1154	$\frac{1}{2}$ v.H.
20.....	0.410	0.0108	0.0270	0.0052	0.0022	0.4552	2 v.H.
21.....	0.207	0.0027	0.0136	0.0026	0.0022	0.2281	-50% K
22.....	0.209	0.0041	0.0103	0.0026	0.0022	0.2282	-24% K, -21% Ca
23.....	0.208	0.0055	0.0103	0.0026	0.0022	0.2286	-21% Ca
24.....	0.212	0.0139	0.0022	0.2281	Na+Ca only
25.....	0.220	0.0057	0.0022	0.2279	Na+K only
26.....	0.058	0.145	0.0022	0.2052	Ca+K only
27.....	0.198	0.0053	0.0200	0.0025	0.0022	0.2280	+53% Ca
28.....	0.196	0.0080	0.0200	0.0024	0.0022	0.2286	+48% K, +48% Ca
29.....	0.194	0.0104	0.0200	0.0023	0.0022	0.2289	+92% K, +48% Ca
30.....	0.182	0.0240	0.0180	0.0022	0.0022	0.2284	+344% K, +33% Ca
31.....	0.187	0.0246	0.0123	0.0024	0.0022	0.2285	+350% K
32.....	0.191	0.0201	0.0125	0.0024	0.0022	0.2282	+270% K
33.....	0.198	0.0052	0.0130	0.0100	0.0022	0.2284	+284% Mg
34.....	0.189	0.0050	0.0125	0.0196	0.0022	0.2283	+650% Mg

the sodium bicarbonate, the concentration of which was constant. Also, with the exception of solutions 19 and 20, osmotic pressure was essentially the same, since the total molarities were relatively constant.

The absence of magnesium from van Harreveld's solution (solution 3) produced no significant effect for short periods up to 2 hours; after that time, decreases in frequency and increases in amplitude occurred at a greater rate than with the whole solution. Obviously, the magnesium ion plays a relatively minor role in sustaining and regulating the heart beat of crayfish. Absence of calcium (solutions 1 and 25), however, caused quick

arrest in systole; absence of potassium (solutions 2 and 24) or of sodium (solution 26) caused quick arrest in diastole. Conversely, isotonic calcium chloride or magnesium chloride caused arrest in diastole, while isotonic sodium chloride or potassium chloride or sodium bicarbonate caused arrest in systole. These results, in some respects, are similar to those reported by Walzl (1937) for the oyster but differ considerably from those of Lindeman (1928) on the crayfish. Since the latter's solution was so incorrectly balanced for calcium, the results obtained by changing from it to other solutions were undoubtedly abnormal results of hearts treated earlier with unbalanced solutions as previously described.

Isotonic sodium chloride regularly caused an immediately increased amplitude and frequency for from 1 to 5 minutes, accompanied by a gradually increased tone and decreased amplitude. During the next few minutes the frequency slowly decreased while the tone increased and the amplitude decreased, until arrest usually occurred in systole in from 9 to 12 minutes, although a few hearts stopped in diastole. Hearts which had been arrested by other means could often be temporarily revived by isotonic sodium chloride (cf. Rogers, 1905). Isotonic glucose (0.45 molar) gave somewhat variable results. In ten hearts a markedly decreased frequency and a gradually increased tone were followed by arrest in systole or near systole within 5 minutes. In three hearts the frequency, the amplitude, and the tone decreased, and arrest occurred in diastole. In one heart the frequency and amplitude decreased, but the tone remained unchanged up to arrest in diastole. Similarly, mixtures of isotonic glucose and sodium chloride gave equivocal results as far as arrest was concerned. However, the typical sodium chloride effect of initially increased frequency and amplitude occurred in mixtures containing 25 per cent of the normal sodium chloride content or more, while a decreased frequency and amplitude occurred in mixtures containing less than this proportion.

By decreasing the molarity of van Harreveld's solution one-half, without altering the ionic ratios (solution 19), marked increases in frequency and amplitude with a slight increase in tone occurred, followed by gradual fatigue. Amplitude decreased faster than frequency, and the beat continued for as long as 20 minutes, when arrest in systole occurred. Recovery in van Harreveld's, however, was incomplete, indicating a certain amount of irreversible injury. Doubling the molarity of van Harreveld's solutions (solution 20) without changing ionic ratios caused quick arrest in diastole, with good recovery in van Harreveld's within a few minutes.

The other solutions tested showed the following qualitative results. The effects produced on the heart were in all cases roughly proportional to the amount of change in cation concentration, the greater effects being produced by the larger changes. Excess of sodium or deficiency of potassium (solutions 4, 14, 21, and 22) caused increased frequency and slightly increased amplitude; excess of potassium or deficiency of sodium (solutions 5, 7, 9, 10, 13, 29-32) caused decreased frequency and increased amplitude; excess of calcium (solutions 8-10, 12, 13, 17, 27-30) and magnesium (solutions 33 and 34) caused decreased frequency and amplitude; a deficiency of calcium (solutions 11, 14, 18, 22, and 23) caused increased frequency and amplitude.

Quantitatively, the most important ionic ratios are those of potassium and of calcium to sodium. As indicated earlier, the magnesium content is of little importance unless a large excess is present (284 and 650 per cent in solutions 33 and 34). Decreasing potassium by 50 per cent (solution 21) caused only a slightly increased rate; increasing it 17 per cent (solution 5) caused no effect. Small changes in calcium, however, caused notice-

able effects (7 per cent decrease in solution 11, 21 per cent decrease in solution 23, and 9 per cent increase in solution 12). Table 3 gives the qualitative effects of increasing the number of potassium and calcium ions per 100 sodium ions in those solutions in which the magnesium and the sodium ions were not significantly changed. Expressed on the basis of 100 sodium ions, the normal content is 2.63 potassium ions and 6.58 calcium ions. The former can probably change by 0.75 (nearly 30 per cent), but the latter by only about 0.25 (about 4 per cent) without causing changes in the heart beat for several minutes. The limits of calcium are, therefore, very narrow; of potassium considerably wider; of sodium and magnesium still wider. The large increases in potassium (270 and 350 per cent in solutions 32 and 31) caused a marked decrease in frequency and a significant increase in amplitude, the latter being followed in from 2 to 5 minutes by a gradually de-

TABLE 3
THE EFFECTS OF INCREASING K AND Ca RATIOS (RELATIVE TO
Na=100), IN SOLUTIONS USED ON THE CRAYFISH HEART

Solution	K	Percentage of Change	Ca	Percentage of Change	Effects on Heart
2.....	0	6.37	Increased rate and tone on fresh hearts
21.....	1.31	-50	6.55	Slightly increased rate on fresh heart
v.H.....	2.63	6.54	"Normal"
1.....	2.63	0	Increased rate; systolic arrest
23.....	2.64	4.94	-25	Increased rate
11.....	2.62	6.04	-8	Increased rate
18.....	2.63	6.23	-5	Slightly increased rate
v.H.....	2.63	6.58	"Normal"
12.....	2.66	7.24	+10	Decreased rate
17.....	2.62	7.92	+20	Decreased rate
27.....	2.67	10.1	+53	Decreased rate
8.....	2.62	12.2	+82	Decreased rate and amplitude
5.....	3.00	+17	6.54	No real effect
7.....	4.85	+84	6.56	Decreased rate
32.....	10.5	+300	6.55	Decreased rate and increased amplitude
31.....	13.2	+400	6.58	Decreased rate; increased tone and amplitude

creasing amplitude and increasing tone. Feeble and slow contractions, however, might continue up to 10 minutes before arrest occurred in systole. In view of the fact that excess potassium "narcotizes" the peripheral nerves of crustaceans (Cowan, 1934), the continued beating of the crayfish heart in solutions 30, 31, and 32 may indicate at least temporary independence of nervous control.

SUMMARY

1. Van Harreveld's solution is far more satisfactory for perfusing the crayfish heart (*Cambarus clarkii*) than Lindeman's or double Ringer's solution. Hearts have continued beating for as long as 20 hours in van Harreveld's.

2. Moderate changes in pH (from 6.4 to 8.4) and slight changes in osmotic pressure do not affect the heart for several hours. Halving the osmotic pressure causes increased frequency and amplitude; doubling it causes quick arrest in diastole.

3. The heart beat is primarily controlled by the sodium ion, which in the absence of other cations causes too high a frequency and amplitude leading to quick fatigue.

4. Potassium, calcium, and magnesium retard the frequency, and a proper balance between them and sodium is necessary for continued beating.
5. The calcium ion is by far the most important antagonist to sodium. Its optimum concentration can be changed by only about 5 per cent without affecting the heart.
6. Increased frequency is caused by excess of sodium or by a lack of potassium, calcium, or magnesium, and decreased frequency by a lack of sodium or by an excess of potassium, calcium, or magnesium.
7. Increased amplitude is caused by excess of sodium or potassium, and decreased amplitude by excess of calcium or magnesium.
8. Arrest in systole is caused by isotonic NaCl, KCl, or NaHCO₃, and arrest in diastole by isotonic CaCl₂ or MgCl₂.
9. Isotonic glucose is more harmful than isotonic NaCl, usually causing arrest in systole or near systole, although a few hearts were arrested in diastole. Mixtures of isotonic glucose and sodium chloride containing 25 per cent or more of the normal NaCl content produced the typical sodium chloride effect of initially increased frequency and amplitude. Mixtures containing less than 25 per cent of the normal NaCl content caused decreased frequency and amplitude, the effect typical of pure glucose solutions.
10. The perfusion solution for the crayfish heart must correspond closely in its inorganic content with the blood serum of the crayfish, if normal behavior of the heart is to be maintained. Simplified solutions may profoundly alter the heart beat, even though it may continue for several hours.
11. The heart may adapt itself to an incorrect solution and continue beating for a considerable time, but its reactions to changes in the perfusing solution may be quite different from those secured following perfusion by a different initial solution.

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SEROLOGICAL INVESTIGATION OF RODENT RELATIONSHIPS

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IT WAS the purpose of this investigation to study the precipitin reaction with anti-rodent rabbit sera, to determine the serological relationships of a series of rodents, and to test the applicability of the reciprocal reaction when the antibody-producing agent (rabbit) belongs to an order of mammals (Lagomorpha) presumably closely related to the order (Rodentia) which supplies the antigen.

Nuttall (1904) employed the precipitin reaction in extensive experiments on the serological relationships of animals. He concluded that "the degree and rate of blood reaction appear to offer an index of the degree of blood relationship." The results of his tests were in general accord with the accepted zoölogical classification.

Nuttall and subsequent investigators studied the serological relationships of representatives of practically all the mammalian orders except the rodents, which were omitted mainly because of the technical difficulty of obtaining suitable anti-rodent sera. The use of fowl as antibody-producing agents results in cloudy antisera (Nuttall, 1904; Boyden, 1926; Hicks and Little, 1931). Earlier investigations (Nuttall, 1904; Boyden, 1926) seemed to indicate that rabbits are unsuitable for developing rodent antisera of sufficient potency for use in determining serological relationships, possibly because of the close relationship between the two. However, Uhlenhuth (1905) was successful in producing anti-hare serum in rabbits, and Hicks and Little (1931) were able to produce antibodies in rabbits against rats and mice in 25 per cent of their attempts. Erhardt (1931), in an extensive review, cited very few investigations with anti-rodent precipitins, and these accomplished little beyond differentiating between the blood sera of rats and mice.

MATERIALS AND METHODS

Antigens used in this work were the blood sera from various rodents. Some sera were obtained from blood samples collected in the field, others were from laboratory stock. In the latter instances blood was obtained by cardiac puncture under ether anesthesia after the manner of Hicks and Little (1931). The blood of the smaller animals was pooled. The blood was centrifuged, and the supernatant serum was pipetted off and stored in the refrigerator in sterile sealed ampules. Aseptic precautions were observed throughout. The blood collected under field conditions was centrifuged, and the supernatant serum removed and passed through sterile Seitz filters before storage.

Standard 1 per cent antigen dilutions were prepared from the whole serum by using sterile 0.85 per cent NaCl buffered to neutrality with Sørensen's phosphate buffers as recommended by Evans (1922). These standard solutions were passed through sterile Seitz filters and stored in small sealed ampules.

Normal, healthy rabbits were used as precipitin-producing agents. Each animal was injected intravenously with 0.2 cc. of whole serum daily for six or seven days. After a rest period of one week another similar series of daily injections was given. Eight to ten days after the last injection, 50-60 cc. of blood were removed by cardiac puncture,

under ether anesthesia. The blood was defibrinated and centrifuged, and the supernatant antiserum was stored in sterile-sealed ampules in the refrigerator.

Occasional attempts to increase the titer of an antiserum by giving a third series of injections were usually unsuccessful in increasing the homologous titer, though the heterologous titers might be altered. This agrees with the findings of Wolfe (1935, 1936), whose results on the effect of injection methods in altering specificity prompted us to give five of the rabbits a third series of six daily injections starting one to two days after bleeding. Eight to ten days after the last injection 50–60 cc. of blood were again removed, and the antiserum was obtained and stored for comparison with the antiserum from the first bleeding of the same rabbits.

In performing the precipitin tests, the "ring-test" method was used because it was found to be much more constant in results than the original flocculation test (unpublished data; cf. also Boyden, 1926).

Preliminary tests were first made on each antiserum to determine its homologous titer. For this purpose an arithmetically progressing series of dilutions was prepared from the standard 1 per cent antigen with sterile buffered saline. In small sterile test tubes 0.2 cc. of antigen dilution was overlaid on 0.1 cc. of homologous antiserum. The tubes were incubated in a water bath for 1 hour at $37.5^{\circ}\text{C} \pm 0.5$. The highest antigen dilution in which a white ring was noticeable at the antigen-antiserum interface was considered the homologous titer of the antiserum.

In performing the actual experimental tests, standard solutions of the homologous and various heterologous sera were diluted according to one or another of a set of arithmetic progressions. These progressions were so arranged that each homologous series usually contained the same number of dilutions regardless of the homologous titer of any antiserum. Thus, if the preliminary titer was about 1:25,000, a dilution series was used which increased by steps of 1:4,000; if the preliminary titer was about 1:20,000, the dilution series increased by steps of 1:3,000. A series increasing by steps of 1:2,500 was employed when the preliminary titer was less than 1:15,000. This method should facilitate comparison between antisera of different titers.

The tests were set up and incubated as described above. The end-point titers of the heterologous antigens were recorded and compared with the homologous end point. As a control, a tube containing 0.1 cc. of antiserum overlaid with 0.2 cc. of buffered saline was incubated with the others. The control was always clear. The possible observational error in reading the end-point titer was found to be \pm one tube within the limits of the dilutions used in this study.

EXPERIMENTAL DATA AND RESULTS

Production of rodent antisera.—Table 1 records the twenty-four antisera produced, the number of injections, the total amount of antigen injected, and the homologous titer of each. There is considerable variability in antibody production; some antisera react strongly with the homologous antigen, others not at all. Furthermore, this variability in homologous reaction is not correlated with the amount of antigen injected or with the injection method, but appears to be an individual reaction of the rabbit. For example, Rabbits 12, 14, and 21 show little change in *homologous* titer upon repeated injections. Rabbits 17, 18, and 23a, treated identically, differ greatly in homologous titer.

Effect of antigen aging upon the precipitin test.—Since some of the sera had been kept 3 years, it was advisable to test the effect of storage. It was found that standard anti-

gens prepared from serum 3 years old and those from freshly prepared serum of the same species reacted to the same degree, within the limits of the observational error, with any one antiserum. The same results were observed with standard solutions prepared 6 months apart from the same serum sample. This constancy of reaction of different sera

TABLE 1
PRODUCTION OF RODENT IMMUNE RABBIT SERA

Rabbit No.	Antiserum For	No. of Injections	Total Injection (Cc.)	Homologous Titer
Q*	Deer mouse	11	5.5	1:30,000
T	Deer mouse	21	6.0	1:1,000
U	Porcupine	17	3.2	1:18,000
V	Wood rat	20	4.1	1:15,000
1	Porcupine	32	6.3	1:21,000
2	Woodchuck	25	4.9	1:20,000
3	Pocket gopher	13	2.6	0
4	Wood rat	13	2.6	1:24,000
5	Woodchuck	13	2.6	1:18,000
6†	Deer mouse	14	2.8	1:28,000
7	Porcupine	14	2.8	1:18,000
8†	Pocket gopher	12	2.4	0
9†	Woodchuck	12	2.4	1:15,000
10	Cotton rat	12	2.4	1:28,000
12a	Muskrat	12	2.4	1:7,500
12b	Muskrat	18	3.6	1:10,000
13	Roof rat	12	2.4	1:10,000
14a	Deer mouse	12	2.4	1:28,000
14b	Deer mouse	18	3.6	1:28,000
16	Woodchuck	12	2.4	1:15,000
17	Porcupine	12	2.4	1:15,000
18	Wood rat	12	2.4	1:32,000
19	Roof rat	9	1.5	1:15,000
20a	Muskrat	12	2.4	1:1,000
20b	Muskrat	18	3.6	1:7,500
21a	Woodchuck	12	2.4	1:18,000
21b	Woodchuck	18	3.6	1:18,000
23a	Porcupine	12	2.4	0?
23b	Porcupine	18	3.6	0?

* Injected with a mixture of powdered egg albumin solution and deer-mouse serum in the hope that the heightened reaction resulting from the development of a high-titered anti-egg albumin serum would induce an increase in the titer developed against the deer-mouse serum (cf. Zinsser, 1931, p. 474).

† Injected with citrated plasma. The reactions of this antiserum were quite comparable with the reactions obtained with antisera from serum-immune rabbits.

‡ Injected with 50 mg. of vitamin C (crystalline Merck), dissolved in 0.85 per cent NaCl, just prior to each daily injection of serum (Jusatz, 1936). No increase in antibody production was noted.

from the same species was apparent throughout the whole investigation. Barring pathological conditions, blood sera of varying ages from the same species can be expected to react to the same degree with a given antiserum.

Effect of aging of antisera upon specificity.—Three antisera (Rabbits U, V, and 2) which had been stored for 60, 80, and 45 weeks, respectively, were tested for comparison with the reactions previously obtained with the same antisera after storage 2–7 weeks. There was a marked increase in specificity as manifested by a retention of the original homologous titer and reduction in the heterologous titers; the weaker heterologous reac-

tions disappeared entirely. Similar observations were reported by Wolfe (1933). Retention of a homologous reaction is no indication of retention of relative specificity. From the foregoing results it is evident that, in order to test the relationships of various heterologous sera to a homologous serum, the tests should be performed with antisera of about the same age.

Effect of variation in total nitrogen content of antigens.—In view of the fact that antigens for the precipitin test are sometimes diluted in terms of their nitrogen content, it seemed desirable to determine the nitrogen content of the antigens employed in the present work. Total nitrogen determinations were made by the Pregl (1930) micro-Kjeldahl method on most of the 1 per cent standard solutions from seven of the antigens used.¹ With one exception the total nitrogen content did not vary widely from species to species. Table 2

TABLE 2

VARIATION IN TOTAL NITROGEN OF 1 PER CENT STANDARD ANTIGEN SOLUTIONS
PREPARED FOR EACH SPECIES AT DIFFERENT TIMES

All tests were performed in duplicate. The values are the averages of such duplicate tests and represent mg. of nitrogen per 5 cc. of 1 per cent solution.

Species	No. of Solutions Tested	Highest Determi- nation	Lowest Determi- nation	Difference	Average Determi- nation
Deer mouse	6	0.360	0.220	0.140	0.285
Pocket gopher	3	0.275	0.155	0.120	0.197
Wood rat	1*	0.597	0.540	0.057	0.568
Woodchuck	2	0.234	0.199	0.035	0.217
Porcupine	1*	0.195	0.192	0.003	0.193
Cotton rat	1	0.190
Muskrat	1	0.152

* Two determinations made on different days on portions of the same standard serum solution.

gives the average reading for each species and also records for each species the variation in nitrogen content of standard solutions prepared at different times. The standard solutions were prepared from pooled sera in the cases of deer mouse, pocket gopher, and cotton rat. The muskrat, porcupine, and wood rat are each represented in the table by the serum of a single individual, though serum from more than one individual of the latter two species was used in the precipitin tests. The two standard solutions of the woodchuck were prepared from the sera of two different individuals, respectively.

It will be noted from Table 2 that, with the exception of the wood-rat serum, the differences between species are not greater than the differences which may arise between determinations of different samples from the same species. Thus, the difference between the averages of deer mouse and muskrat is 0.133 mg., all the other interspecific differences being less, except for the wood rat. It will be seen from the fifth column of the table that variations between serum samples prepared at different times from a single species may easily equal or surpass such interspecific differences. These variations within a species may be due to variation in the accuracy of pipetting in the preparation of stand-

¹ We wish to express our appreciation to Miss Thera Visscher, technical assistant in the Department of Zoology, who performed these tests.

ard solutions, to error in the micro-Kjeldahl test, to possible fluctuations in concentration of serum proteins, to differences in non-protein nitrogen due to the varying metabolic states of the animals at the moment of bleeding (particularly when pooled sera are not used), or to a variety of other factors.

The reason for the much higher nitrogen content of the wood-rat serum is not evident. It is of interest to note that this greater nitrogen content did not result in the production of antisera of consistently higher titer than was elicited by antigens of lower nitrogen content (Table 1). No nitrogen determinations were made on the samples of whole serum used for the inoculation of rabbits, but there is no reason to doubt that their nitrogen concentrations were proportional to those of the dilute solutions on which such determinations were made.

TABLE 3
PRECIPITIN REACTIONS OF CLOSELY RELATED RODENT SERA WITH
ANTISERA OF DIFFERENT SPECIFICITIES

Rabbit	Homologous Titer	Deer Mouse	Wood Rat	Cotton Rat
7.....	1:18,000	1:9,000	1:12,000	1:6,000
12a.....	1:7,500	1:1,000	0	0
12b.....	1:10,000	1:10,000	1:10,000	1:7,500
17.....	1:15,000	1:1,000	1:3,000	1:1,000
19.....	1:15,000	1:3,000	1:6,000	1:1,000
20.....	1:7,500	0?	1:1,000	1:1,000
21.....	1:18,000	1:3,000	1:3,000	1:3,000
		Gray Squirrel	Woodchuck	
6.....	1:28,000	1:1,000	1:1,000	
7.....	1:18,000	1:1,000	1:6,000	
10.....	1:28,000	1:4,000	1:1,000	
12b.....	1:10,000	1:1,000	1:1,000	
14a.....	1:28,000	1:1,000	1:4,000	
14b.....	1:28,000	1:4,000	1:8,000	
18.....	1:32,000	0	1:4,000	

The greater nitrogen content of the wood-rat standard solutions did not appear to modify the values obtained in the precipitin tests. From the upper portion of Table 3 it is evident that with a variety of antisera the three closely related forms—deer mouse, wood rat, and cotton rat—gave reactions of closely similar magnitude. The standard solution of wood-rat serum included in Table 2 and an additional one were used for the tests included in Table 3; the two reacted similarly. Most of the differences in end point shown in Table 3 are within the observational error of \pm one tube (see above). For example, in but two cases was there a difference of two tubes between the end points with wood-rat antigen ($N=0.593$ mg.) and those with cotton-rat antigen ($N=0.190$ mg.).

It seems evident that, within the limits of the series of forms here employed, variation between species in total nitrogen content of normal serum does not form a significant variable as compared with the magnitude of the other variables involved.

Influence of increased antigen injections upon specificity.—The precipitin reactions of the antisera from two different bleedings of five rabbits (see Table 1) were compared.

One (Rabbit 23) failed to produce any measurable titer at either bleeding; another (Rabbit 21) gave identical reactions with the antisera from both bleedings. Of the remaining three (see Table 4), Rabbits 12 and 14 revealed a loss in specificity, evidenced by increase in heterologous titers with tendency to retain homologous titers. This agrees with the results of Wolfe (1935, 1936, 1939) and corroborates his conclusion that, when comparable results are desired, as in determining zoölogical relationships, a uniform injection procedure should be maintained. The loss in specificity was less apparent in the two bleedings from Rabbit 20, since the first bleeding yielded a very weak antiserum. It is interesting to note that the antiserum from the second bleeding of Rabbit 20 had the same order of specificity as the antiserum obtained from the first bleeding of Rabbit 12. This seems to indicate that rabbits may differ in speed of antibody production as well as in strength of antibody production.

Tests of rodent relationships by means of the precipitin reaction.—The taxonomic relationships of the rodents studied in this work² are given by Miller (1923) as follows:

ORDER RODENTIA

- I. Superfamily Sciuroidae
 - A. Family Sciuridae
 - 1. Subfamily Sciurinae
 - a) *Marmota monax* (woodchuck)
 - b) *Sciurus carolinensis* (gray squirrel)
 - B. Family Geomyidae
 - Geomys bursarius* (pocket gopher)
- II. Superfamily Muroidae
 - A. Family Cricetidae
 - 1. Subfamily Cricetinae
 - a) *Peromyscus gossypinus* (deer mouse)
 - b) *Sigmodon hispidus* (cotton rat)
 - c) *Neotoma albigula* (wood rat)
 - 2. Subfamily Microtinae
 - Ondatra zibethica* (muskrat)
 - B. Family Muridae
 - Rattus rattus alexandrinus* (roof rat)
- III. Superfamily Hystricoidae
 - A. Family Erethizontidae
 - Erethizon dorsatum* (porcupine)

The rabbits and hares were formerly regarded as constituting a suborder within the order Rodentia. Mammalogists now include them in a separate order (Lagomorpha), which is usually placed immediately following order Rodentia in the classification (for status of the group see Gidley, 1912). The exact interrelationship of the two orders has not been determined. Nevertheless, it seems probable that the rodents are more closely related to the lagomorphs than are the other orders of mammals, although there is not unanimity of opinion on this point.

The results obtained with nineteen antisera against seven of the nine rodents studied

² We are indebted for material to Dr. Lee R. Dice and Dr. Philip Blossom, of the Laboratory of Vertebrate Genetics, University of Michigan; to Dr. Robert B. Aiken, Mr. Herbert Daigneault, Mr. Robert Fyfe, and Mr. Avery King.

are shown in Table 4. Three attempts to produce anti-pocket-gopher serum failed; limited supply of serum prevented attempts to produce anti-squirrel serum.

Because of possible effects on specificity, injection methods were kept constant (see Table 1), and tests were performed with antisera 7-12 weeks old. Even under these conditions antisera of different specificities were produced. For example, antisera from

TABLE 4
RESULTS (IN PERCENTAGE OF HOMOLOGOUS REACTION) OF PRECIPITIN TESTS EMPLOYING
VARIOUS RODENT ANTISERA TESTED WITH A SERIES OF RODENT SERA

RABBIT	ANTISERUM	ANTIGENS								
		Deer Mouse	Wood Rat	Cotton Rat	Roof Rat	Musk-rat	Porcupine	Woodchuck	Gray Squirrel	Pocket Gopher
Q.....	Deer mouse	100	83	50	33
6*.....	Deer mouse	100	71	71	43	14	0	4	4	4
14a*.....	Deer mouse	100	71	71	43	29	4	14	4	14
14b.....	Deer mouse	100	100	100	57	43	14	29	14	14
V.....	Wood rat	83	100	50
4*.....	Wood rat	67	100	4	0	4
18*.....	Wood rat	75	100	75	50	38	38	13	0	3
10*.....	Cotton rat	71	71	100	29	29	14	4	14	4
13.....	Roof rat	0	0	0	100	0	0	0
19.....	Roof rat	20	40	7	100	7	0	0	0
12a.....	Muskrat	13	0	0	0	100	0	0	0	0
12b.....	Muskrat	100	100	75	100	100	10	10	10	0
20a.....	Muskrat	0	0	0	100
20b.....	Muskrat	0?	13	13	0	100	0	0	0	0
1*.....	Porcupine	14	71	100	29	5
7*.....	Porcupine	50	67	33	50	17	100	33	6	0
17.....	Porcupine	7	20	7	0	7	100	0	0	0
2.....	Woodchuck	5	50	100	0
5.....	Woodchuck	0	0	0	0	0	0	100	83	0
16.....	Woodchuck	0	0	0	0	0	7	100	100	0
21*.....	Woodchuck	17	33	17	0	6	33	100	100	6

* Antisera used in Table 5.

a Antisera from rabbits after two injection series.

b Antisera from the same rabbits after a third injection series.

Rabbits 14a and 10 reacted with all the heterologous sera, while that from Rabbit 13 reacted only with the homologous antigen; various intermediate degrees of specificity occurred.

Despite these differences of specificity among the antisera, the results indicate a serological relationship among the rodents. With few exceptions the relative positions of the heterologous sera to the homologous serum are the same with antisera against the same species even though the actual quantitative results differ widely. The exceptions involve apparent reversals in the relative positions of some of the weaker heterologous re-

actions. It may be noted that most of these reversals include porcupine serum or antiserum, though the significance of that fact is not yet apparent.

Analysis of the serological relationships shows striking agreement with the accepted taxonomic relationships. With deer-mouse antisera (Table 4) the largest heterologous reactions occurred with wood-rat and cotton-rat sera, both of which are regarded as different genera within the same subfamily as the deer mouse. Next in magnitude were the reactions with roof rat and muskrat, which are in the same superfamily as the deer mouse. The weakest reactions involved rodents morphologically more distantly related to the deer mouse. Reactions with wood-rat and cotton-rat antisera revealed the same relationships; deer-mouse, wood-rat, and cotton-rat sera reacted most strongly, followed by roof-rat and muskrat reactions. Two roof-rat antisera (Rabbits 13 and 19) differed widely in specificity; one reacted only with the homologous serum, the other with the four more closely related heterologous sera. Agreement between the serological and morphological relationships was still apparent, since the heterologous reactions included only members within the same superfamily as the roof rat. Essentially similar results were obtained with anti-muskrat sera.

The results with anti-porcupine sera are of doubtful significance in determining serological relationships because of the shifts already noted in relative position of the heterologous reactions from antiserum to antiserum.

With the woodchuck antisera the strongest heterologous reaction occurred with gray-squirrel serum; these two forms are regarded as related genera within the same subfamily. With woodchuck and porcupine antisera there is one marked difference between the serological results and the accepted classification. With woodchuck antisera, porcupine serum reacted more strongly than did pocket-gopher serum; with anti-porcupine sera the woodchuck reacted more strongly than did the pocket gopher. This points to a closer relationship between the woodchuck and the porcupine than between either and the pocket gopher. Yet, the woodchuck and pocket gopher are regarded as members of the same superfamily, while the porcupine is placed in a distantly related superfamily. Because of the doubtful value of the porcupine reactions, however, definite conclusions must await further investigation.

Application of the reciprocal reaction.—Boyden (1926, 1934) formulated the principle of reciprocal relationships in serological investigations: "The percent values of the relationships between any two species A and B should be of the same order of magnitude, whether antiserum A is tested with antigen B or antiserum B is tested with antigen A." However, he found that, with antisera built up in rabbits, the proper relationship of rodents to other animals could not be determined, owing to an inhibition of rabbit response to rodent antigens. This lack of reaction was not apparent with antisera produced in fowl. Therefore he stated, "The principle of reciprocal relationships holds only when the antisera are produced by animals which are about equally distant from the species tested." Reciprocal testing with anti-rodent rabbit sera is in accord with this reasoning, since any two rodents, as contrasted with a non-rodent, are about equally removed from the rabbit.

Owing to the extreme differences in specificity, it is obvious that positive reciprocal reactions could not be obtained if all the antisera were compared. Table 5 records the reciprocal tests for five rodents obtained with eight antisera which appeared to have the same degree of specificity (checked with an asterisk in Table 4). Of ten reciprocals, six were definitely positive within the possible observational error (\pm one tube), one (wood-

chuck vs. wood rat) was doubtful, and three involving porcupine reactions were doubtful or negative. This is further indication that the porcupine reactions are aberrant and of doubtful value for determining serological relationships. The results indicate that the reciprocal test is valuable in checking relationships among the rodents *if antisera are compared which have the same order of specificity*.

Another possible method of checking the reliability of the precipitin reaction is suggested by the assumption that antigens which are closely related serologically should react to about the same degree with any antiserum regardless of its specificity (Wolfe, 1933). To illustrate this point, we may cite results with deer mouse, wood rat, and cotton rat, and with squirrel and woodchuck. The tests show that the first three are closely

TABLE 5
RECIPROCAL REACTIONS (IN PERCENTAGE OF HOMOLOGOUS REACTION) WITH
ANTISERA OF THE SAME ORDER OF SPECIFICITY

Homologous Antigen	Heterologous Antigen	Per-centage Reaction	Homologous Antigen	Heterologous Antigen	Per-centage Reaction	Diff.
Deer mouse	Wood rat	71*	Wood rat	Deer mouse	71*	0
Deer mouse	Cotton rat	71*	Cotton rat	Deer mouse	71	0
Deer mouse	Porcupine	4	Porcupine	Deer mouse	32*	28x
Deer mouse	Woodchuck	9*	Woodchuck	Deer mouse	17	8
Wood rat	Cotton rat	75	Cotton rat	Wood rat	71	4
Wood rat	Porcupine	21*	Porcupine	Wood rat	69*	48x
Wood rat	Woodchuck	13	Woodchuck	Wood rat	33	20?
Cotton rat	Porcupine	14	Porcupine	Cotton rat	33	19?
Cotton rat	Woodchuck	4	Woodchuck	Cotton rat	17	13
Porcupine	Woodchuck	31*	Woodchuck	Porcupine	33	2

* Average of results from two antisera.

x Negative reciprocal reaction.

? Doubtful reciprocal reaction.

interrelated, as are the last two. Table 3 records the reactions of these animals with antisera of varying specificities. The closely related rodents reacted to the same degree, within the possible observational error, in ten out of fourteen cases. Of the four negative reactions, none differed by more than two dilution tubes.

DISCUSSION

Nuttall (1904) was of the opinion that a powerful anti-rodent serum, if obtained, would react generally with all rodents, since the lack of reaction of rodent sera with non-rodent antisera suggested that the rodents were a closely related serological group. But subsequent results have not confirmed this opinion. Hicks and Little (1931) found serological differences among four species within the genus *Mus*. Avrech and Kalabuchov (1937) reported differentiation between species and even subspecies within the mouse genus *Apodemus*. Wolfe (1939) was able to distinguish between genera within the family *Sciuridae*. In the present work closely related genera within the same subfamily were

differentiated. It is possible that species within a genus might have been distinguished with some of the very specific antisera if the proper antigens had been available.

Differentiation between rodents is to be expected when rabbits are the antibody-forming agents. The rodents and rabbits are presumably closely related and possibly possess some serum proteins in common. Since antibodies are formed against foreign proteins, it can be understood why rabbits should produce specific anti-rodent sera. Landsteiner (1936, p. 13) observed:

Apparently, the degree of relationship between the animal species furnishing the antigen and that used for immunization has considerable influence on the results . . . rabbit immune sera are very suitable for revealing dissimilarities in the proteins of other rodents, while in the case of birds, if rabbit sera are employed, the lesser differences will be hidden by structures common to bird proteins.

Supportive evidence was given by Hicks and Little (1931), who were unable to distinguish between rat and mouse sera by use of pigeon-immune sera, though they succeeded with rabbit-immune sera.

Injection methods and the age of antisera were shown to affect their specificity. It is important that these factors be kept constant where comparable results are desired. Even when these conditions are controlled, however, antisera of widely differing specificities are produced. Such differing specificities may be due to intrinsic differences in the rabbits producing the antisera, or to some property of the injected serum manifested by apparent individual variation of response among the rabbits. Relative to this second possibility it may be noted that Heidelberger and Kendall (1935) gave successive courses of injections of purified egg albumin to rabbits and noted progressive changes "believed to consist in the formation of more and more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule." Hooker (1937) postulated the existence of multiple antigenic determinants, possessing a possible diversity of antigenicity, upon the same protein molecule. Although blood serum is composed of a complex mixture of proteins rather than of a single protein, these views suggest a possible explanation for the observed differences in the reactivity of the antisera.

In such an antigenic complex as the blood serum, the number of proteins (and/or protein radicals) common to a series of rodents would presumably be in direct ratio to the degree of relationship of the rodents to one another. Closely related rodents would have more proteins (and/or radicals) in common than would distantly related rodents. Individual variation of rabbits in response to the lesser constituents of the serum might loom large in affecting the precipitin reactions subsequently given by an antiserum to distantly related antigens. Such variations might lead to discrepancies and even reversals from antiserum to antiserum. Varying amounts and injection schedules of antigen might also be expected to produce similar variations in the resultant antibody formation against these minor substances.

In spite of these variations in antisera, the results indicated a quantitative serological relationship among the rodents studied. In most cases the relative positions were the same from antiserum to antiserum. A strictly quantitative relationship among the rodents could not be determined, however, because of the extreme differences in antiserum specificity.

Boyden's application of the reciprocal reaction (1934) resulted in a three-dimensioned phylogenetic tree showing the quantitative relationships of five mammalian sera to one

another. The animals furnishing the sera were rather distantly related to one another and also to the antibody-producing rabbits; the quantitative relationships obtained were of a general nature. Wolfe (1933, 1935) could not obtain constant reciprocal reactions with a series of carnivore sera. In the present work it was apparent that the reciprocal reactions would not check if all the antisera were considered. This affords further evidence that individual differences among antisera are conspicuous in work with closely related antigens.

Excellent agreement in reciprocal reactions was obtained, however, when antisera of about the same order of reactivity were tested. Under these conditions reciprocal reactions established the relative relationships of the rodents tested, though the actual percentages obtained were rather a measure of the specificity of the antisera than a strictly quantitative measure of the relationships of the antigens. Even so, since it is found that antisera of like specificity give measurably comparable reciprocal reactions, the reciprocal test is valuable in checking the relationships of even closely related groups. The problem of establishing a truly quantitative phylogeny, as distinguished from a phylogeny of relative position, however, is yet to be solved, at least for such closely related animals as the rodents here employed.

SUMMARY

1. Rodent-immune rabbit sera were obtained by intravenous injections of small daily doses of serum over an extended period of time.
2. The age of an antigen had no effect upon the precipitin reaction.
3. Increased antiserum age resulted in increased specificity as manifested by reduction in heterologous titer, while the homologous titer remained nearly constant.
4. Increased number of antigen injections resulted in decrease in antiserum specificity as manifested by increase in heterologous titer, while the homologous titer remained nearly constant.
5. Within the limits of the series of forms here employed, variation between species in total nitrogen content of normal serum does not form a significant variable.
6. Even when the foregoing conditions were kept constant, antisera of different specificities were produced. Possible explanations are discussed. Such individual differences in specificity appear to form a more significant source of error in investigations involving closely related forms than in studies of more distantly related animals.
7. Positive reciprocal results were obtained if antisera were compared which were of the same order of specificity.
8. Serologically closely related antigens were found to react to the same degree with any antiserum regardless of its specificity.
9. Serological relationships of a series of rodents showed striking agreement with the morphological classification with one possible exception. Rodents of different superfamilies were separated very readily; families and subfamilies within the same superfamily were distinguished from one another; in many instances it was possible to differentiate closely related genera within the same subfamily.

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MODIFICATION OF THE SOCIAL ORDER IN FLOCKS OF HENS BY THE INJECTION OF TESTOSTERONE PROPIONATE¹

(One figure)

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MODERN scientific recognition of the existence of a definite social organization within small flocks of birds dates from the work of Schjelderup-Ebbe (1922) on the so-called "peck-order" in the common domestic fowl (*Gallus domesticus*). He has also observed the phenomenon which he calls "despotism" in a large number of birds, and he summarized his work on the subject in 1935. Schjelderup-Ebbe's original description for chickens² has been essentially verified, among others, by Masure and Allee (1934) and by Murchison in his 1935 series of papers.

This social organization is based primarily on the outcome of an initial pair-contact, or a series of such contacts, in which one bird loses a fight or submits passively without fighting. Thereafter, when these two fowls meet, the one which has acquired the peck-right (that is, the right to peck without being pecked in return) exercises it frequently. The subservient bird of an original pair often avoids close contact with her superior and, in event of a revolt, usually does not again fight back vigorously. Revolts occur rarely among hens, and successful revolts that result in a change in social status are still more rare.

Factors which make for dominance between members of the same sex and species of birds have been reported to include the following (see Schjelderup-Ebbe, 1935; Shoemaker, 1939):

1. Other things being equal, the stronger bird usually wins.
2. One bird acts as though intimidated by the appearance of a strange and apparently unfrightened individual and so gives way without fighting.
3. Both birds act as though frightened, but one recovers more rapidly and so wins the contact reaction.
4. An individual which is normally aggressive and victorious may be temporarily out of condition (tired or ill or molting severely) when it meets a newcomer, and so loses when it might, from other relations, be expected to win.
5. Mature hens usually dominate immature chickens.
6. The location of the first contact is important; birds, like many mammals, fight better in their home territory.
7. Even in territory strange to both members of a contact pair a bird wins more readily if surrounded by others with which it has associated.

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² The *Standard Dictionary* defines "chicken" as "the young of the common fowl; loosely, a fowl of any age. . . ." The word is used here in this looser sense.

8. Birds with young fight more fiercely than they would at others times. With some birds—canaries, for example—intensity of fighting varies with the different phases of the reproductive cycle.

Two main types of social order have been described for birds. Among chickens, for example, once social dominance is established, it tends to be maintained unless there is a decided revolt. The dominant birds seem to have an absolute peck-right over their subordinates. With several other species—of which pigeons, doves, and shell parakeets may be mentioned—the result of any given pair-contact can seldom be predicted with certainty, even though, in the long run, one of the pair wins more frequently than does the other. Masure and Allee (1934) called this a "peck-dominance," in contrast with the peck-right found among chickens.

It is readily seen that with animals that have an almost absolute peck-right any reverse pecks which may be observed are much more significant than they would be if the society were organized about peck-dominance. Hence, for the present studies the use of the common domestic fowl was indicated. Also, since cocks have a more unstable peck-order than hens, the latter were used exclusively.

A description based on the pecks administered or received does not give a very intimate picture of the actual social relations in a flock. As a result of observing birds, all of which are plainly marked to permit individual recognition, one finds definite personality traits which are not revealed by the more formal statistical summaries of the results of pair-contacts. A given individual may be hesitant, apparently fearful, or aggressive in different degrees; and its behavior may or may not vary in relation to the different social ranks of its associates. Dominant hens are characterized by the lack of any attempt to avoid other members of the flock; they may ignore the others, or they may chase and peck them. Only exceptionally are they pecked in return.

Subordinates low in the social order tend to avoid birds of higher rank. They retreat when pecked or threatened by high-ranking birds, and may give distinctive sounds, as though in pain, when pecked severely. The lowest-ranking birds in three of the first four pens observed in this study showed objective signs easily interpreted as fear of their superiors. Such behavior depends, often, but not always, on the behavior of the superior birds and grades from a mere quickening of pace to a strong avoiding reaction, which may include dodging backward, turning, and speedy retreat with some vocalization, as though in fear.

The severity of the peck, when given, varies widely. Blood may be drawn from the comb; feathers may be plucked; or, on the other hand, the peck may be light. The attack is often aimed at the comb or the top of the head; but, as the subordinate usually dodges the blow, it frequently lands on the neck or even on the back or shoulders or not at all. Under these conditions the superior hen often renews the attempt to deliver an effective blow. The fowls are usually close together when pecking, but threats may be given from a distance of even 2 feet or more. Pecks are rarely attempted when the subordinate is too far away to be hit.

Pullets and hens do not always react to each other when they come close together. They may be feeding busily about the food hopper and may pay no attention to the presence of another individual. Similarly, when sunning or otherwise resting during the day and after they settle on the roost at night, pecking is usually suspended. Frequently contacts can be stimulated by placing some mixed grains or similar food among the litter on the floor of the pen.

Some birds, usually high in the pecking-order without being at the top, show little negative reaction to their superiors and, when pecked, generally give only a slight avoiding reaction. Some individuals react strongly to being pecked. They may retreat to the other end of the pen, the roost, or the top of the nest boxes, or may hide in some corner, stand still with the head held low, or even crouch low with wings half lifted and the tail depressed, i.e., squatting, as hens do before a rooster. It would be easy to read human emotions into these reactions, a temptation which not all writers on the subject have resisted successfully.

Revolts and reverses are rare among hens. When an inferior pecks a superior, the latter, if it reacts at all, appears "surprised" rather than "frightened." It does not retreat, except to gather itself for attack; it draws itself up, leaps forward, and administers vigorous return pecks. The subordinate hen, which initiated the revolt, retreats as a rule but may also stretch up as high as possible, in characteristic fighting pose. Then each hen may leap forward, beak toward beak with neck elongated and arched and neck feathers raised, and so attempt to peck down on the head or seize the comb of its opponent. Noble, Wurm, and Schmidt (1938) in their study of the social organization of the black-crowned night heron report what may be a foreshortened variant of this type of behavior.

The frequency of pair-contacts varies greatly with different individuals and also with the time of day for the same birds. Lorenz (1935, p. 361), speaking presumably of jackdaws, says that there is a certain tension between birds which stand close to each other, particularly between those which stand near the top animal, and "on the other hand birds high in the peck order are good-natured to the low ranking birds." We have some evidence that this is true in our flocks. With some exceptions, we have found that hens high in the social order tend to peck their more immediate inferiors more often than they do those lower in social rank. A good part of the explanation, but not all, is that the lower birds, especially those near the bottom of the peck-order, tend to avoid their dominant pen mates when these come too near. Then, too, birds that rank near together are more often in close competition with each other.

Some of the individual differences mentioned in the preceding paragraphs are illustrated by observations made in preparation for the injections to be reported below. In one pen, BB, the α -bird pecked its subordinates an average of 41 times each over a period of 42 days; while in another pen during the same time, BR, also an α -bird, pecked its inferiors an average of 126 times. BR had fewer subordinates to peck; and one of them, GG, always kept away from the mash hoppers until the other birds had left. Another evidence of the difference between these two top birds was that BR frequently drove all other birds away from the mash hopper in the mornings; BB was not seen to do this.

Other complexities, which are poorly revealed in the simple tabulation of pecks, include cases of apparent antipathy between individuals. In one of the preliminary studies, BR pecked GG 131 times and its only other subordinate, which, furthermore, was its immediate inferior, only 64 times, although it had more opportunity to peck the latter, since GG, the bottom bird of its flock, commonly kept at some distance from the other birds. RW was seen to peck RY 122 times, while during the same observation periods it pecked RR only 57 times; yet RR stood just below RY in social rank. This could be interpreted as an example of the suggestion made by Lorenz which has just been cited; if so, the α -birds are able to draw a fine line in their behavior toward closely ranking subordinates. Discrepancy of treatment is also indicated by the observation that some superiors allowed some inferiors, but not all, to peck food from their bills.

Sickness limits the social activity of a bird and may result in a loss in social position. Unfortunately, we have had a considerable amount of experience with this factor. Of the original 24 top-crossed pullets which were received October 19, 1937, only 3 are now living. The majority of those that died have been autopsied by the veterinary department of Iowa State College,³ and, of these, almost all died of fowl leucosis, with which the animals were infected when first received.

Evidence has been accumulating of a partial hormonal control of the aggressive reactions on which the social position of birds is based. This led Allee in 1936 to suggest the use of sex hormones in analyzing the underlying physiological basis of the social order in flocks of birds. Briefly, some of the evidence is: With domestic fowls, and with many other dimorphic species in which the male is larger and more showy, the male normally stands above the females in the social order. Domm and Van Dyke (1932) had found that injection of hebin, an extract of the anterior pituitary which contains LH and FSH as well as thyrotropic hormone, into young male leghorns causes great precocity in the development of head furnishings and also induces crowing (9 days), fighting, and treading by the age of 13 days. Capons certainly do not fight as vigorously as do cocks; in fact, Domm's experience (1927) led him to believe that a "gonadless" male neither crows, treads, nor fights. Benoit (1929) cites a decided exception to this general rule, and one of us has seen brief capon fights in Professor Koch's capon colony. Shoemaker, as early as 1936, had evidence that the social position of an individual canary tended to change during the progress of its breeding cycle.

These and other indications suggested that female aggressiveness might be enhanced by the injection of male hormone. The work of several investigators had shown (see Witschi and Miller, 1938, for a summary) that hens produce male hormone; and Gustavson (1932) prepared an extract from hen feces which stimulated comb growth in capons, a reaction now generally agreed to be produced only by androgenic stimulation. The injection of more male hormone would not mean the introduction of a totally strange material into the female system.

EXPERIMENTAL MATERIAL

Four lots of pure-bred white leghorn pullets or hens have been used in these studies. Their constitution and previous history follows:

Flocks A 1, A 2, A 3, and A 4.—These were top cross white leghorns procured from Dr. N. F. Waters, of Iowa State College. Each of these A flocks was composed of full sisters, the offspring of a random-bred hen mated with an inbred sire. The inbreeding coefficients of the sires used were 52.5, 67.0, 52.1, and 76.8 per cent, respectively. This means that the amount of inbreeding ranged from approximately four to something less than eight generations of brother-sister matings. Their breeding history is given by Waters and Lambert (1936). These pullets were approximately 6 months old when received in October, 1937. They were housed, 6 to a pen, in newly rebuilt and carefully fumigated pens in the Whitman Laboratory chicken-house. The pens were 14.5 feet long by 4.5–5.0 feet wide and about 8.0 feet high. Each pen is provided with raised roosts over a solid board platform to catch the droppings. The cement floor of the pens is kept covered with litter, which is changed weekly. Small doorways open onto sun porches on the south side of the buildings. These porches have wire bottoms as well as sides and top, so that the animals are held well above the ground. Light comes to the

³ We are indebted to Professor N. F. Waters and to Dr. E. F. Waller for making and reporting these autopsies.

pens from a window at the south end of each; and, in addition, a 60-watt bulb hangs over the center of each pen. These are operated on a time switch and provide extra illumination during morning and evening hours during the winter. Winter temperature was controlled by a thermostat and ranged between 10° and 15° C. Our four pens were completely separated from other parts of the chicken-house and were on separate thermostatic and light controls. Our compartment was entered from the outside by its own door, which opened into a narrow corridor that gave direct access to each pen.

Each bird carried numbered wing and leg bands. For our purposes these were supplemented by one or more colored leg bands the colors of which gave the designations of the different individuals. GY, for example, wore a green and a yellow leg band. The subscript which is added at times indicates the flock number.

Almost immediately after receipt, flocks of the A series were placed under observation to determine the normal peck-order. These preliminary observations lasted from October 24 to December 24, 1937. Between that date and April 7, 1938, they were used in a set of experiments to determine the effects of injection of epinephrine.

As a result primarily of fowl leucosis, only 16 of the original 24 birds were alive on April 8. At or before that date the remnants were combined into two flocks, which were appropriately designated "A 1, 2" and "A 3, 4." The former contained 9 and the latter 7 individuals; further deaths reduced these numbers to 6 and 5, respectively, by the end of the first experiments with testosterone propionate, hereafter referred to as TP.

Flocks A 1, 2 and A 3, 4 were given some weeks under daily observations until the peck-order became stable. Injections of TP lasted from June 6 through July 29 (54 days in all), after which postinjection observations were continued until August 16. The 9 healthy birds still remaining were combined into flock A 5 and watched for 2 weeks; and selected birds were injected for 51 days, beginning August 30. After 3 weeks of postinjection observation only 5 were left. These were combined with 2 old hens recently received from an Indiana flock that had been line bred for 18 years without the introduction of new stock. This combination will be called flock "A 6."

Flocks B 1 and B 2.—These were originally composed of 24 inbred white leghorns from Iowa State College equally divided between their families 4 and 6 (Waters and Lambert, 1936). The individuals hatched out between January 3 and 31, 1938, and were received by us in two lots on March 19 and April 9, respectively, when they were 2–3 months old. Family 4 had at that time an inbreeding coefficient of between 68.0 and 73.6, and Family 6 had one of 59.3. Each of our B flocks contained 6 pullets from each family. In 1936 the birds in these two families had an *inter se* relationship of 62; the full sibs of Family 4 had a relationship of 88; and of Family 6, one of 74. The B flocks were housed 12 together in pens vacated by combining the A flocks. They were placed under observation immediately on receipt in order to study the establishment of the peck-order. Half of flock B 1 was removed for ovariectomy on June 22 and 23 and was permanently returned to the flock on July 19.

Injections of TP were made into selected individuals of flock B 2 from October 19 to December 21, 1938. On January 13, 1939, only 6 of this flock were still alive. Those autopsied showed no fowl leucosis. There were diagnoses of ulcer of the gizzard or proventriculus or erosion of the gizzard (4 cases) and an infected air sac (1 case). In flock B 1, 4 operated and 4 unoperated controls were alive on the same date. Those autopsied showed: acute hepatitis with fatal hemorrhage from rupture of the liver; abscess on breast muscle and a discolored liver, apparently secondary to abscess, since this bird was never injected:

Flocks C 1, C 2, and C 3.—These were also composed of pure but random-bred white leghorns. They were battery reared in our laboratory from newly hatched chicks which we received September 19, 1938, from the Illinois hatchery, of Metropolis, Illinois. Later they were housed 6 to a battery 40 inches long by 24 inches wide by 18 inches high (C 1 and C 2) and six more in one 28 inches long by 22 inches wide by 24 inches high (C 3). The former were kept at 20°–21° C.; the latter at 6° C. They had not been used in previous experiments before the present work began. The chickens received TP injections from December 10, 1938, to January 30, 1939.

Flock D 1.—This flock was originally composed of 12 aged hens of which 2 were taken to make flock A 6. They were received October 22, 1938. Something of the ruggedness of the stocks and the adequacy of our treatment is seen by the fact that, beyond the death of 1 bird in each of the C and D flocks almost immediately on receipt, all the rest were alive and apparently well in late February, 1939.

Care and feeding.—The A, B, and D flocks were fed and cared for by Mr. Collias except for brief periods, when Miss Lutherman acted as substitute. These two also made all the primary observations. While no other person has handled or cared for these flocks, Dr. L. V. Domm and his experienced caretaker, Mr. Emil Stange, have been extremely courteous in keeping watchful eyes on the welfare of our chickens and in giving advice concerning their care. The diet and treatment have been carefully based on the experience of Dr. Domm with his much more extensive colony.

Briefly, they were fed moist mash containing cod-liver oil in the morning and coarser, mixed grain in the afternoon. A hopper of dry mash was always present in each pen. Green food (cabbage or lettuce) was constantly present off the floor at the side of the pen. Water was before the birds at all times, and the containers were cleaned daily. Oyster shell, crushed limestone, and charcoal were always available to the birds.

The C flocks have been constantly under the care of Miss Lutherman. They have been fed Conkey's starting mash, growing mash, and mixed grains and have also had water constantly before them. In place of cod-liver oil they have been irradiated from 1 to 2 hours daily with light containing about the amount of ultra-violet found in noon sunlight.

Methods of observation.—In the A and B flocks behavior records throughout have been made by an observer sitting quietly, notebook in hand, in a neighboring pen, partly screened from the sight of the birds by the solid wooden partition that makes up the lower part of the walls of each pen. Only large-meshed chicken wire was present to interfere with the view of the near-by birds. Although easily disturbed when first placed under observation, the successive flocks were readily conditioned to the presence of the observer, as was shown by the resumption of normal activities even in the pen in which the observer was seated.

All pair-contacts were initiated by the birds themselves without stimulation other than by the fresh distribution of food. During the period of these experiments the birds were under observation 3–5 hours daily except that on Saturdays, when the pens were cleaned, and on Sunday observations were curtailed or omitted. Observations were made during one or two morning periods between 8:00 and noon and an afternoon period between 2:00 and 6:00. The B flocks were observed by the same method; also, they, together with flocks A 5 and A 6, were placed together in a neutral pen, pair by pair, for observation of initial contacts. The details of this procedure will be given later. Flocks C 1 and C 2 were watched from a distance of 4–6 feet; under the conditions that prevailed one could see the bands on the birds in the two middle batteries. So far, up to 6 months

of age, it has been possible to observe all birds of both pens at once and to record all pecks. Periods of observation of these C flocks have varied from 15 minutes to an hour, with 15-20 minutes per day being the more usual time spent. The first peck was observed when the birds were about 2.5 months old. During the early weeks, before pecking started, there was little to observe that is of interest from the present point of view. Filling the feed troughs just before the observation period is of little importance with these battery-housed birds, since they do not "fight" over the opportunity to feed. It was difficult to make accurate observations in the cold room because of the small space there.

OBSERVATIONS

Flock A 1, 2.—As constituted on April 8, this flock contained 9 hens. These were observed for 7 weeks before TP injections were begun; during the last 6 weeks of this preliminary period the social order had remained stable and is summarized in Table 1. This

TABLE 1
THE SOCIAL ORDER IN FLOCK A 1, 2 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked							
BB	8	RG	RR	GG	BR	YY	BY	GY	RW
RW	7	RG	RR	GG	BR	YY	BY	GY
GY	6	RG	RR	GG	BR	YY	BY
BY	5	RG	RR	GG	BR	YY
YY	4	RG	RR	GG	BR
BR	3	RG	RR	GG
GG	2	RG	RR
RR	1	RG
RG	0

flock had organized itself in a pure-line system or simple hierarchy. The birds indicated by bold-faced type were selected for injection with the male hormone.⁴ All injections were given daily and were made intramuscularly into the pectoral muscle. The locations of the punctures were varied systematically. The hormone was administered as received in sesame oil, and all other birds in the same flock were similarly injected daily with 0.1 or 0.2 cc. of sesame oil. In the present experiment **RG** received 1 mg. and **BR** 0.5 mg. of TP daily. As a check on the effectiveness of the hormone, Dr. L. V. Domm kindly consented to make independent injections of our TP into one of his long-time capons. According to expectations, there was prompt and vigorous comb growth.

Four of the hens died during the period of the experiment: BB on June 7, of fowl leucosis; GG on June 19, of impaction of crop and gizzard, RR on July 31, of fowl leucosis; and GY on August 1 (no diagnosis). **BY** died approximately 3 weeks after injections stopped. The reversals over these birds that died and even over **BY** may have been made more certain by reason of the ill-health of the losing bird. This cannot always have been

⁴ The testosterone propionate (TP) was donated by the Ciba Pharmaceutical Products Company, Lafayette Park, Summit, N.J., and by the Schering Corporation, Bloomfield, N.J. Without their kind co-operation this work could not have been done. We detected no difference between the hormone furnished by the two companies:

an important factor, however, for BB maintained her position at the top of the pre-injection order up to her death. The ranking of the reduced flock on August 1, 2 days after injections stopped, is given in Table 2.

The basic observational data on which earlier tables are based are given in Table 3 in the form of summaries of the observed pecks for stated intervals which are arbitrarily chosen to give the maximum of information without making the table too long and complicated. Another observer was added during the fourth week after injections were started. The unsuccessful revolts that were observed are shown by asterisks, and the approximate date of each reversal can also be estimated. None of the reversals was actually observed. All the return pecks that are recorded for the first period actually were given on the first day that flocks A 1 and A 2 were united. The dramatic thing shown by the tables is the rise of RG from the bottom to the top position in the flock. This hen stood fifth among the 5 survivors of flock A 1. There is evidence that it did not submit passively to the lowly position which it occupied in flock A 1, 2. After the first day, however, no

TABLE 2
THE SOCIAL ORDER IN FLOCK A 1, 2 AFTER 54 DAYS OF INJECTIONS

Individual	Number Pecked	Individuals Pecked				Former Rank*	Formerly Pecked*
RG	3	BY		YY	RW	5	0
RW	3	BY	BR	YY		1	4
YY	2	BY	BR			3	2
BR	2	BY			RG	4	1
BY	0					2	3

* Among those still living

overt indication of revolt was seen in the long period before injections started, and it was not until these had been going on for 4 weeks that RG began its social climb. BR successfully revolted against GY almost 5 weeks before the death of the latter, against BY 6 weeks before she died, maintained her dominance over RG, and staged one observed unsuccessful revolt against RW.

Flock A 3, 4.—On March 19 the remnants of former flocks A 3 and A 4 were combined into a flock of 7 individuals. No reversals in position occurred in the 11 weeks preceding injection of male hormone, which, as with the preceding flock, began on June 6. The dominance-subordination pattern of the flock at that time is shown in Table 4. The social order in this flock illustrates another basic type from that shown in Table 1. Here the top bird, GY, dominated all the others except RG, which, although it stood third from the bottom, had managed to get and hold superiority over GY. As shown again by bold-faced type, the lowest-ranking hens were injected daily with TP, GG with 0.5 mg. and BG with 0.25 mg. As always, the others were similarly injected with sesame oil. Two birds died during the injection period;⁵ the ranking of the remaining members of the flock on August 1 is shown in Table 5.

Again no successful revolts were actually seen, but the daily observations permitted these to be dated with confidence. The unsuccessful revolts that were observed and the

⁵ RG on June 16, of fowl leucosis; GY on July 19, no diagnosis but with "no enlargement of organs as expected in leucosis."

TABLE 3

SUMMARY OF OBSERVED PECKS IN FLOCK A 1, 2 BEGINNING APRIL 8

Hens injected with hormones and pecks in the reversed direction are shown in bold-faced type. Double vertical lines inclose the period of injection. Asterisks mark unsuccessful revolts.

DOMINANT	SUBORDINATE	DAYS SUMMARIZED								PECKS	RE-VERSED PECKS
		10	21	28	14	14	14	12	18		
RW.....	GY	1	4	8	25	15	11	3	Dead	67	0
	BY	3 6	*10	17	4	3	5	5	13	63	3
	YY	2	3	3	3	1	5	4	9	30	0
	BR	1	12	14	*13	13	11	1	19	84	2
	GG	2	8	8	6	Dead	24	0
	RR	1	11	5	9	5	Dead	31	0
GY.....	RG	1 1	12	4	0	4	1	2	62	22	65
	BY	1	3	4	15	8	0	6	37	0
	YY	6	2	6	14	3	0	4	GY	35	0
	BR	5	2	8	4	1 46	26	8	Dead	20	80
	GG	5	1	1	3	Dead	10	0
	RR	1	4	1	24	1	Dead	31	0
BY.....	RG	6 2	0	2	*7	6	1	3	19	10
	YY	14	23	15	38	15	5 9	15	8	110	32
	BR	9	14	8	8	8	12	1	9	47	22
	GG	7	11	2	0	Dead	20	0
	RR	5	24	9	24	7	Dead	69	0
	RG	1 8	7	10	6	5	1 26	11	20	37	8
YY.....	BR	5	36	5	19	10	20	12	10	117	0
	GG	3	16	6	9	Dead	34	0
	RR	6	8	8	13	2	Dead	37	0
	RG	3 2	8	6	9	5	3 19	25	58	33	105
	GG	3	11	12	2	Dead	28	0
	RR	4	34	26	37	8	Dead	109	0
BR.....	RG	2	13	18	6	27	3	1	6	76	0
	GG	3	31	4	5	GG	Dead	43	0
	RG	2 2	13	12	0	27	2
RR.....	RG	2	1	3	8	1	RR	Dead	16	0
RG.....	0	0	0	0	0	0	0	0	0	0
Totals.....	127	323	227	311	194	158	101	214	1,276	379

TABLE 4

THE PECK-ORDER IN FLOCK A 3, 4 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked						
GY.....	5	BG	GG	BY	BR	RR
RR.....	5	BG	GG	RG	BY	BR
BR.....	4	BG	GG	RG	BY
BY.....	3	BG	GG	RG
RG.....	2	GG	GY
GG.....	1	BG
BG.....	1	RG

other basic data are summarized in Table 6, as in Table 3. In this flock again both injected birds moved up in the social scale: GG went from next to the bottom to the top,

TABLE 5
THE PECK-ORDER IN FLOCK A 3, 4 AFTER 54 DAYS OF INJECTIONS

Individual	Number Pecked	Individuals Pecked				Former Rank*	Formerly Pecked*
GG.....	4	BY	BR	BG	RR	4	1
RR.....	3	BY	BR	BG	1	4
BG.....	2	BY	BR	5	0
BR.....	1	BY	2	3
BY.....	0	3	2

* Among those still living.

TABLE 6
SUMMARY OF OBSERVED PECKS IN FLOCK A 3, 4 BEGINNING MARCH 19

Hens injected with hormone and pecks in the reversed direction are shown in bold-faced type. Asterisks mark unsuccessful revolts. Double vertical lines inclose the period of injection. BG was added on April 30.

DOMINANT	SUBORDINATE	DAYS SUMMARIZED									PECKS	RE-VERSED PECKS
		8	28	14	28	14	14	14	12	18		
GY.....	RR	19	17	15	47	29	46	16	GY	189	0
	BR	18	17	20	19	11	*20	7	Dead	112	0
	BY	12	20	4	17	25	16	11	105	0
	GG	15	13	7	21	10	*16	*10	92	0
	BG	16	41	19	18	35	129	0
RR.....	BR	***30	33	18	27	16	13	2	11	22	172	0
	BY	40	24	19	33	41	38	18	19	2	234	0
	RG	31	32	9	38	19	Dead	129	0
	GG	18	17	15	24	21	15	*9	7	113	126	113
	BG	27	65	22	55	76	82	55	382	0
BR.....	BY	6	10	5	6	7	*1	12	15	8	70	0
	BG	24	51	25	21	12	Dead	134	0
	GG	5	17	8	17	18	*1 22	21	37	57	67	137
	BG	36	57	19	3 11	18	1 33	1 52	117	114
BY.....	RG	20	15	3	6	14	Dead	58	0
	GG	0	0	3	1	*8	12	2	8	23	12	43
	BG	2	5	4	22	44	54	5	11	125
RG.....	GY	24	21	5	21	22	RG	93	0
	GG	14	10	7 1	*19	18	Dead	68	1
GG	BG	39 2	46	28	23	14	25	40	215	2
BG	RG	2	10	20	Dead	32	0
Totals.....	276	297	288	542	383	332	295	293	376	2,547	535

and BG staged successful revolts against BY and BR. It is worth noting that GY maintained its dominant position until its death and was able to put down attempted revolts which were made on three different occasions by GG; also, that BR was broody during

the injection period, and its comb, and presumably its supply of male hormone, became much reduced.

Flock A 5.—This flock was composed of the 9 remaining birds from the original A flocks. They were placed together on August 17 and were watched then for the first 5 hours, which is the period most crucial for the establishment of the social order. As it happens, in this flock there were no reversals after this initial period for the first 4 weeks that

TABLE 7
THE SOCIAL ORDER IN FLOCK A 5 BEFORE TREATMENT

Individual	Num- ber Pecked	Individuals Pecked								
RG.....	7	BY	RY	BG	RR	GG	YY	RW
RW.....	7	BY	RY	BG	RR	GG	BR	YY
YY.....	6	BY	RY	BG	RR	GG	BR
BR.....	6	BY	RY	BG	RR	GG	RG
GG.....	4	BY	RY	BG	RR
RR.....	3	BY	RY	BG
BG.....	2	BY	RY
RY.....	1	BY
BY.....	0

TABLE 8
THE PECK-ORDER IN FLOCK A 5 AFTER 51 DAYS OF TREATMENT OF
THE HENS SHOWN IN BOLD-FACED TYPE

Individual	Num- ber Pecked	Individuals Pecked									For- mer Rank	Former- ly Pecked
BY.....	7	BG	RR	GG	BR	YY	RW	RG	9	0
RG.....	6	BG	RR	GG	RY	YY	RW	1	7
RW.....	6	BG	RR	GG	RY	BR	YY	1	7
YY.....	4	BG	RR	GG	BR	3	6
BR.....	4	RR	GG	RY	RG	3	6
RY	4	BG	RR	YY	BY	8	1
GG.....	3	BG	RR	RY	5	4
RR.....	1	BG	6	3
BG.....	1	BR	7	2

the hens were together—and then only by injected individuals. All the birds that received testosterone gave 1 or more reversals. There were no deaths during the progress of the experiment. The peck-order on August 30, when injections began, is shown in Table 7.

BG was given 0.75 mg., RY 1.00 mg., and BY 1.25 mg. of TP daily. BG was removed on October 4 (ninth period) because of illness, and her injections were discontinued. Otherwise the hormone was administered for 51 days. The flock organization at that time is shown in Table 8. Again the dramatic feature of the experiment is the climbing of BY from the bottom to the top of the flock, where she dominated all except RY, which

had also received substantial injections of male hormone. **RY** went up from eighth place to a tie for fourth, with reversals over **RR** and **YY** and **BG**. Despite illness, **BG** lost only to her pen mates that were also receiving the hormone, and gained a reversal over **BR**.

Two successful revolts were observed. On September 13 **RY** attacked her superior, **YY**; they fought briefly, and **YY** was beaten and retreated. Thereafter **RY** frequently asserted her superiority over **YY**. Fifteen days later **RY** engaged in a more elaborate encounter with her superior, **RR**. She fought with **RR** just after the latter was returned to the pen following injection with sesame oil. **RR** turned away and retreated, an action which marks a reversal if the fight is decisive. A little later **RY** leaped on **RR**, and they fought vigorously for a moment, then **RR** ran. **RY** kept picking up and dropping a small feather and calling. Later **RY** again jumped on **RR** in fighting pose and pecked her severely; **RR** retreated and ran. Still later **RY** gave **RR** another vigorous peck, and this time **RR** flew to a roost. After a time **RR** came down to the floor only to receive another peck from **RY**. All this happened between 4:00 and 4:30 P.M. Although these two birds had not been seen to fight that morning, **RR** had threatened **RY** during the earlier observation; hence, this probably was the complete story of the reversal, while the action between **RY** and **YY** may have been merely the final scene in a longer struggle, the early phases of which were not observed.

An unexplained pair-contact reaction was seen on October 10 at about 5:00 P.M. Earlier in the period of watching, **GG** had pecked **RY**, as was usual; then near the end of the observation period, **RY** and **GG** fought actively for 5 or 10 seconds, during which time **RY** seized **GG** by the comb and threw her down. **GG** got away and retreated beneath the roosts, where she remained for some time. **RY** did not follow but flew to the top of the nest boxes and stayed until the observer left. Apparently this had been an entirely successful revolt, but the next day **GG** was again pecking **RY** as though nothing had happened. The fact that the revolt occurred late in the day and that **RY** did not immediately follow up her advantage may have had something to do with the matter. Of course, there may have been another unwitnessed and crucial encounter which **RY** lost, especially since later observations of another reversal, not in connection with this experiment, showed that a series of revolts occurred before the dominant bird relinquished the peck-right to a revolting subordinate. During the series of combats still another bird, dominant to both, kept joining in and fighting against the initial rebel, almost as if aiding the defending dominant bird, which, nevertheless, in this case eventually lost its position. This illustrates one of the unavoidable difficulties of our work; in order to know all that happens, the hens should be under constant surveillance and yet, as has been indicated earlier, there would be hours and hours of such watching that would ordinarily be wasted.

The basic observations with flock A 5 are summarized in Table 9, which is arranged as were Tables 3 and 6.

Since the other experiments to be reported involved other stocks of birds, at least in part, and used other techniques, a summary of the effects produced in these tests which were made on the A flocks is given in Table 10.

During the same period of time, of the 12 individuals similarly injected with sesame oil alone, only 1 reversal was given. This was by **YY** over **BY** in flock 1, 2 and took place after **BY** had lost to its 2 hormone-injected flock mates; **BY** died just after the close of the experiment. Of the 22 reversals given by the hens receiving hormones, only 5 are less important, because they were made over a bird which may have been ill. On the

other hand, BG in flock A 5, the only one of these injected individuals to become ill while receiving hormone, gained and kept 1 reversal shortly before she was removed because of illness.

TABLE 9

SUMMARY OF OBSERVED PECKS IN FLOCK A 5 BEGINNING AUGUST 17

Hens injected with hormone and pecks in the reversed direction are shown in bold-faced type. Double vertical lines inclose the period of injection. Asterisks mark unsuccessful revolts.

DOMINANT	SUBORDI- NATE	DAYS SUMMARIZED								PECKS	RE- VERSED PECKS
		1	12	13	7	7	7	17	18		
RG.....	RW	22	20	5	2	1	0	6	1	57	0
	YY	17	25	26	4	11	11	5	8	107	0
	GG	1	8	12	15	9	2	16	3	66	0
	RR	17	8	9	1	6	4	3	48	0
	BG	9	19	16	10	13	13	7	0	87	0
	RY	1	11	10	8	4	1	*11	6	52	0
	BY	13	12	30	15	*6	1 98	62	77	160
RW.....	YY	7	2	13	2	10	7	20	8	69	0
	BR	9	6	8	14	27	6	36	5	111	0
	GG	7	6	5	12	1	1	20	5	57	0
	RR	12	*9	11	3	2	4	2	1	44	0
	BG	7	1	11	5	4	8	2	0	38	0
	RY	12	5	10	5	2	1	15	5 3	55	3
	BY	10	3	14	27	16	5	8 42	34	83	76
YY.....	BR	7	15	13	2	18	31	65	13	164	0
	GG	3	1	0	0	5	1	36	5	51	0
	RR	5	13	6	0	3	4	3	2	36	0
	BG	8	*8	5	5	13	10	9	6	58	0
	RY	4	11	3	3 4	21	13	27	18	21	83
	BY	4	2	3	2	13	3	60	27	11	103
	RG	2	3	12	5	4	4	22	1	53	0
BR.....	GG	4	15	13	2	9	18	82	13	156	0
	RR	44	9	*5	2	6	16	12	4	98	0
	BG	9	6	7	1 1	2	8	1 1	0	26	10
	RY	7	14	7	3	23	24	47	17	142	0
	BY	4	25	12	25	35	9 1	102	52	110	155
	RR	5	3	12	8	8	17	3	2	64	0
	BG	1	15	6	21	48	14	0	105	0
GG.....	RY	2	7	16	17	21	*46	15	124	0
	BY	4	6	29	14	16	133	19	198	23
	BG	2	3	3	4	19	5	0	36	0
RR.....	RY	5	2	*7	7	12 8	3	6	33	17
	BY	3	5	*6	9	10	5	2	23	17
	RY	2	5	3	2	6	23	1	0	12	30
BG.....	BY	4	3	8	1	4	0	0	15	5
RY.....	BY	3	7	7	10	14	71	62	174	0
BY.....	0	0	0	0	0	0	0	0
Totals.....	222	299	310	293	365	405	571	402	2,661	682

In general, as Table 10 shows, there were more reversals as the dosage became larger. BG again is an exception on this point; it was also the only individual receiving male hormone which allowed other hormone-injected birds to rise above it in social status. Both

BR in flock A 1, 2 and **RY** in flock A 5 retained the peck-right over their injected associates **RG** and **BY**, respectively, which otherwise went to the top of their social groups.

As the more detailed tables show (3, 6, and 9), there was a latent period before the injected birds began to stage revolts, and the beginning of reversals came still later. Reversals were usually made first over the fowls low in the peck-order, and only near the end of the prolonged injection periods, if at all, over the ranking birds in the respective flocks.

As was to be expected, TP produced other physiological effects besides those reflected directly by changes in social position. Certain of these will be discussed later, when all such effects will be considered together.

Flock B 2.—These birds were placed under continuous daily observation about the time that they were first beginning to peck each other. During the early months of observation, social position in this particular flock of highly inbred birds was more unstable

TABLE 10
THE EFFECT OF MALE HORMONE ON SOCIAL
POSITION IN THE A FLOCKS

Flock	Bird	Daily Dosage (Milligrams)	Number of Reversals
A 3, 4.....	BG	0.25	2
	GG	0.50	3
A 1, 2	BR	0.50	2
	RG	1.00	4
A 5.....	BG*	0.75	1
	RY	1.00	3
	BY	1.25	7
Totals.....	7	22

* Became ill during experiment.

than that of any other flock of domestic fowls of which we have record. The last spontaneous reversal occurred during the twenty-seventh week of observation; the next to the last happened 4 weeks earlier. Thereafter the social order remained stable. Eleven of the 12 original birds were alive at the beginning of injections. Three control members of the flock and two that were receiving injections (**BB** and **GG**) died during the injection treatment. The peck-order of those that survived throughout and all those injected with TP is shown in Table 11 at the beginning of injections. As before, the individuals listed at the left in bold-faced type were given the male hormone daily; the others received 0.2 cc. of sesame oil at the same time. **GG** was given 1.25 mg. of TP from October 19 through December 4; **BB** received 1.0 mg. daily for the same period. **RW** was injected with 0.75 mg. each day until December 4, when its dosage was doubled for the following 17 days. **GG** and **BB** died about a week after their removal from the experiment on December 4.⁶

No reversals were given by any of the birds in flock B 2 during the period of injection. Four unsuccessful revolts were seen: 3 by birds that were receiving hormone, and 1 by a

⁶ Autopsies at Iowa State College showed erosion of the gizzard and extreme emaciation of both; **BB** also had an egg free in her body cavity.

control. Nineteen reverse pecks were seen; 7 were delivered by 2 of the 3 pullets injected with TP, and 12 by 5 controls. In other effects, as will be seen later, injections in this flock produced physiological results similar to those in the A flocks, and, as will be told in the next section, the injected birds showed definite behavior effects as a result of receiving the hormone. Under these conditions we have no complete and certain explanation of the failure to induce social reversals such as had been given by each of the previously injected birds. Since crowing was induced early in the bird receiving the highest dosage and was discontinued in a short time, it is possible that these young birds became somewhat refractory as a result of continued treatment with heavy dosage. The primary difference between these birds and the others appears to be in age; and it is to be noted that previous investigators (Hamilton, 1938), working with young chicks, did not get a crowing response in the female by injection of male hormone. Since there were no reversals, there is no need to exhibit the basic data. Approximately 4 months later we were able to produce a typical set of reversals in this flock by another series of injections (see p. 434).

TABLE 11
THE SOCIAL ORDER IN FLOCK B 2 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked						
BG.....	7	GG	RW	BB	YY	RR	BR	GY
GY.....	5	GG	RW	BB	YY	BR
RR.....	5	GG	RW	BB	YY	GY
BR.....	4	GG	BB	YY	RR
YY.....	2	RW	BB
BB.....	2	GG	RW
RW.....	2	GG	BR
GG.....	1	YY

INITIAL CONTACTS

The observations reported so far were made by watching the reactions of the birds when engaged in their normal activities in the flocks of which they were constantly members and in which each had a well-established social niche. A revolt or reversal was therefore made in the face of all the social inertia inherent in such a flock organization. As has been shown in the introductory pages, social position among these birds is established by the outcome of initial contacts and, once established, is rarely reversed.

In connection with other studies we devised a system of experimentally induced initial contacts. The typical procedure for Series I was to scatter some scratch food on the floor of the observation pen; then one person caught one of the birds, while another caught the other. Each individual bird came from a separate flock; in the cases we are now considering, only the injected birds had ever been in contact with members of the other flock, and with them 5 weeks or more had elapsed before the contacts were repeated. Each fowl was weighed, the state of its plumage and general health was noted, position in the social order in the home flock was recorded, and standard comb measurements were taken. Length of comb was measured along the greatest length of the blade; height was measured for the point which stood highest and most perpendicularly above the head. Successive measurements on the same individual were made on the same point. Finally

a prediction was entered concerning the outcome of the pair contact which was about to be staged. One observer took a bird under each arm, while the other prepared to make and record observations. The chickens were introduced into a strange pen, which was purposely reduced to half the standard size to insure closer association.

In Series I, five sets of such initial contacts were staged. Set I was between fowls in flock B 2 which had been selected for injection and all those in flock B 1. This latter flock was a wholly similar flock of inbred birds of the same age; the only difference was that 5 of the 10 birds then in flock B 1 were poulards. Set II was between all members of these two flocks and was begun 32 days after injections were started. By that time the comb approached its maximum height. Set III consisted of pair-contacts between the survivors of A 5 and the 2 old Indiana hens which were to be combined with them to make flock A 6. They were given these controlled initial contacts on neutral territory; and, when permanently placed together 5 days after the first pair-contact of the series, they took the position in the social order which was indicated by the outcome of the staged initial encounter. Set IV was a series of staged encounters between members of flock A 6 and the B flocks. Set V includes all staged contacts in the C flocks.

Series II was run by one observer alone with as few modifications as feasible from the routine in Series I. The principal difference was that in the earlier series the same bird was never introduced with another on successive days, while in Series II most birds were so introduced every day. Their opponents, however, always varied from day to day. The contacts in Series II were staged from January 11 to February 27 in connection with studies on the effect of estradiol on the social order and were begun 21 days after the last injection of TP.

Although available in tabular form, the full details will not be given. Many of the data may be used later in another connection, and only those are presented here which bear directly on the role of male hormone in determining social position. Originally this series of observations was begun to test whether there is a relation between the size of the comb among closely inbred normal pullets and the result of the socially important first encounter between two strange birds. The observations were extended to include all other normal individuals together with operated and hormone-injected birds, to allow as complete a test as possible. The results are summarized in Table 12. Some of the general findings are: Poulards with small combs, though often weighing more and standing higher in the social order of their home flock, lost all but 2 encounters. These 2 instances were won by the poulard BB, which had the smaller comb, was heavier, had the higher social rank, and won by passive submission of the other bird.

Pullets injected with TP, though low in social position and light in weight, won all but 4 of their pair-contacts. Such birds had, on the average, larger combs and presumably, therefore, had more male hormone in their system than did the birds to which they lost. The number of actual fights in these staged contacts was small; of the 124 encounters which were staged first, only 24 were settled by fighting. In the remainder, 1 bird retreated without a struggle, i.e., there was passive submission.

In 16 of the 74 cases in which poulards were involved, the bird with the larger comb lost. Two of these involved the poulard BB mentioned above, when for some unknown reason the normal birds submitted passively. The 11 such contacts listed in the table were between the poulard GG and the birds of flock B 2. At the time of these experiments GG resembled a masculinized poulard or a pullet on full injection dosage with TP, since its comb was much larger than that of its pen mates. On closer inspection,

small cocklike hackles could be detected on the neck and flanks, and small spurs were present. Later observations showed continued masculinization.

The contacts between this GG₁ and YY₂ and between GG₁ and BR₂ were unusual in that the first reactions were inconclusive. The third time GG₁ and YY₂ were placed together, the latter won. With BR₂ it was not until the fourth staged encounter that we decided that GG₁ was the loser. The protocols of these encounters are given in Table 13. Both these normal birds acted more warily in the presence of GG than when placed with other birds which they dominated. To human eyes at least, GG was not as aggressive as was to be expected from its appearance. These observations raise another interesting

TABLE 12

THE OUTCOME OF INITIAL CONTACTS ANALYZED ON THE BASIS OF SIZE OF COMB AS AN INDICATOR OF THE AMOUNT OF MALE HORMONE PRESENT

SERIES	CONTACT PAIRS*	BIRD WITH LARGER COMB		MEAN SIZE OF COMB*†	
		Won	Lost	Won	Lost
1.....	Normal:Normal	32	13	128	115
2.....	Normal:Normal	33	12	150	133
1.....	TP:Normal	12	4	143	131
2.....	TP:Normal	22	2	148	133
1.....	TP:Poulard	12	2	156	89
2.....	TP:Poulard	3	0	156	107
1.....	Normal:Poulard	37	11	108	87
2.....	Normal:Poulard	6	3	184	103
2.....	TP:TP	0	2	142	146
2.....	Other combinations	81	35	145	131
Totals or true means.....		238	84	140±0.9	120±0.9

* One pair of birds, not included here, had combs equal in size.

† Length plus height.

possibility. The relatively sudden growth of turgid, hypertrophied furnishings on the head of a relatively slight hen present some handicaps for successful fighting. The delivery of effective pecks is probably rendered more difficult, and the possibility of being pecked on the comb is certainly increased. Many of the problems suggested by the changed appearance of injected hens, or of such operates as GG, both psychological and physiological, could be effectively attacked by the use of dubbed birds.

Of the four contacts lost by hormone-injected birds and won by normal controls, 2 fights each were won by 2 normals from the same 2 injected pullets. These normal individuals (BW and BY) had smaller combs than their injected opponents; they were, however, able and enduring fighters. One of the injected birds (BB) was losing weight rapidly at the time, and the other (GG₂) seemed to be blind in one eye; both were ill and died after about 3 weeks. Neither had the endurance of BW or BY, although each won all its remaining staged contacts with normal birds.

These experimental contacts provide another indication of the increase of aggressiveness in these fowls following TP treatment. **BB**, **RW**, and **GG** of flock B 2 lost 9 of their 12 contacts with **BW**, **RW**, **RR**, and **BY** of flock B 1 before injection started. These were the only ones tried at that time. **BB** and **GG** lost all 4 contacts, and **RW** lost 1 of 4. After 32 or more days of injection, and despite the fact that **BB** and **GG** were not very well at the time, **RW** won all 4 and the others each won 2 of their 4 contacts with the same birds. An adverse score of 9 losses and 3 wins before treatment was turned into a favorable one of 8 wins and 4 losses. Further, the operate **BB**, had won from **BB**, and **GG**, before injections were begun but lost to these as well as to **RW** while they were being given male hormone.

TABLE 13
A SYNOPSIS OF EXPERIMENTAL PAIR-CONTACTS BETWEEN THE POULARD
GG, AND THE NORMAL HEN, **BR**,

	I	II	III
Date.....	10-7-38	10-23-38	10-28-38
Weight, pounds...	3.6:3.6	3.3:3.3	3.2:3.3
Comb, millimeters.	55+101:42+76	56+97:32+67	56+103:30+67
	Large, red:Normal	As before	As before
Social position....	8 :5	As before	As before
Molting.....	No :Yes	As before	As before
Start, P.M.....	4:13	3:53	4:23
Behavior.....	GG avoids BR BR half-threatens GG 4:33, out, no contest 5:04, in again GG avoids BR BR feeds GG avoids BR BR half-threatens GG 5:22, out, no contest	GG feeds GG apparently avoids BR BR half-threatens GG BR feeds BR threatens GG GG feeds 4:13, out, no contest NOTE: BR is pecked by RW , which has large comb, and acts as though afraid of GG 's appearance	BR threatens GG GG avoids BR BR threatens GG 4:27, out BR judged to have won

In general, we can conclude from these observations on the B flocks that, while the injections did not cause the lowest 3 birds to climb in the social order of their home flock, it did decidedly increase their aggressiveness and success in encounters with their near relatives in a parallel flock. Also, these staged initial contacts between normal pullets showed that, while the amount of male hormone present, as measured by comb size, is not an infallible guide to the winner of such contacts, it is a good indicator—in fact, one of the best single indicators we have found—of the probable winner.

The C flocks.—The whole treatment of the C flocks differs radically from that which we have been describing. These were battery reared in our laboratory. One of our interests with them was to study the onset of pecking in two flocks of 6 birds in separate batteries at 20°–21° C., as compared to those in another battery which, since their thirteenth week of age, had been kept at 6° C. The temperatures were chosen in connection with another experiment. The birds were hatched on September 18. **RG**, of flock C, in the warmer room, was seen to peck a battery mate on December 3. A week later, during which time no pecking had been seen, injections of TP were begun into **RR** in the warm room and **GG** in the cool room. **RR** was seen to peck another bird a week later (Decem-

ber 17) and continued to do so steadily. Injections were continued until January 20, 1939, when **RR** became the α -bird of the now firmly established peck-order.

BB of the same flock was first seen to peck another bird on December 17; it was not found pecking again until 4 weeks later. Between December 23 and 29, 5 more of the 11 controls in the warm room were observed pecking other birds. The smaller space in the cold room made observation difficult. No pecks were recorded for these pullets until after injections were begun; and then **GG**, the injected bird, did the first pecking that was seen. **GG** started a few days after **RR** began in the warm room, and has in general behaved much the same as **RR**, at least as nearly as can be detected. We recognize that these observations on the C flocks are inconclusive. For one thing, the injections did not begin until the most precocious bird had started to peck its fellows. However, the behavior is at least what would be expected from the observations on the other flocks.

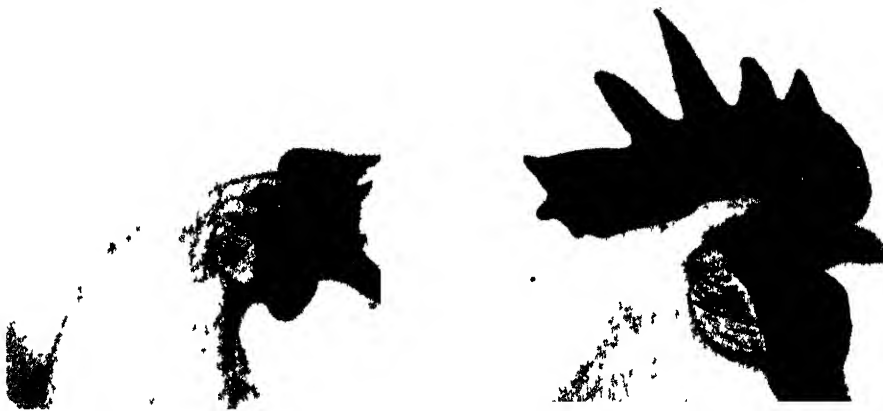


FIG 1—**BY** of flock A 5 before (left) and after 51 daily injections with testosterone propionate

OTHER EFFECTS

Comb growth.—As was to be expected, the comb and other head furnishings of the birds given TP started to grow immediately. A typical effect is given in Figure 1, in which the left photograph shows **BY** of flock A 5 on August 23 before injection with TP and the right photograph shows her on October 19, after having been injected with 125 mg. daily of TP for 51 days. In the meantime, as shown in Table 8, she had moved from the bottom to the top of the social order in her flock. Some general indication of the size of comb attained in comparison with uninjected flock mates is found in the fact that the birds in the A and B flocks showed a mean increase in length plus height of 38 per cent from the beginning to the peak size, while oil-injected controls increased only 2 per cent. A decrease set in at the close of the injections, which in flocks A 1, 2 and A 3, 4 amounted to 3.3 per cent on the sixth day following cessation of treatment. In the A flocks, the full comb size, as measured by length plus width, varied between 197 and 211 mm. The combs of the birds not given male hormone, which ranked highest in the social order of

each flock, ranged from 168 to 186 mm. The combs of flock B 2 were smaller; the three TP-treated pullets at full comb development ranged from 154 to 163 mm. at a time when their pen mates had combs which varied between 86 and 147 mm. The mean percentage increase among these injected birds of B 2 was 55 per cent, as contrasted with an average decrease of 7 per cent in average comb size among their controls. The comb of RW, the one treated bird to survive, decreased 9 per cent in the first 18 days following cessation of injection.

It is possible that the smaller total size of comb which was attained among these injected birds of this highly inbred flock may have been correlated with their failure to move up in the social scale. Against such a conclusion it must be considered that the percentage of difference in comb size between TP-injected birds and their controls was greater in this flock than it was in the A flocks, where each bird that received the hormone did move up in its social ranking and where one injected individual reached top position in each flock.

TABLE 14
EFFECT OF TESTOSTERONE ON WEIGHT

FLOCK	DAYS INJECTED	NUMBER OF CONTROL BIRDS	NUMBER TREATED	CHANGE IN WEIGHT (OUNCES)		PERCENTAGE OF CHANGE	
				Control	Treated	Control	Treated
A 1, 2.....	54	3	2	- 1	+ 8	- 1.6	+12.3
A 3, 4.....	54	3	2	- 1	+ 3	- 1.6	+ 5.0
A 5.....	51	6	2	- 5	+ 0.5	- 7.7	+ 0.8
B 2.....	11	7	3	- 1.1	+ 1.5	- 2.0	+ 2.8
B 2.....	54	5	1	- 3.7	- 1	- 6.7	- 2.0
C.....	42	2	2	+18.5	+11.5	+59	+42

In the C flocks the combs of the 2 hormone-injected pullets increased 200 per cent during the period of treatment (December 10-January 20), and the comb of each showed a very slight but measurable decrease on the day following the cessation of injections.

Weight.—With young chicks Breneman (1939) found that those injected with TP showed no definite weight changes during the period of treatment but that the controls increased more in weight afterward than did the injected birds. On the other hand, Bulliard and Ravina (1938) found that, after 2 or 3 weeks of treatment with the same androgen, ducks about 2 months old showed a slight increase in weight with males and a proportionately greater increase with females.

Our experience on this point is summarized in Table 14, which shows that in the older birds (A and B flocks), in which the animals had wholly or approximately completed their growth when injections of TP were begun, the treated individuals gained weight or in one instance lost slightly, while the controls all lost weight. Considered as 4 paired comparisons, the differences in the first 4 cases have a statistical significance (P) of 0.032. With the young, rapidly growing birds of the C flocks the controls grew more rapidly; and, when these are considered together with the first four sets, the results are statistically insignificant ($P=0.37$). In so far as gain or loss of weight is taken as an indication of well-being, we would expect from these results that the hormone-treated individuals in the

A and B flocks were in better physiological condition than their controls and hence might come to have a higher social status.

Crowing.—Precocious crowing in young male chicks has been induced by the age of 60 hours by the administration of 20 mg. of TP in a single dose on the day after hatching (Hamilton and Dorfman, 1939). This marks a further speeding-up of the crowing reaction from earlier records of 10 or 11 days through the injection of TP (Hamilton, 1938; Breneman, 1938). Hamilton failed to secure crowing with female white leghorn chicks, although they were injected and observed for a period of 55 days. Our experience with the induction of crowing in pullets and hens is summarized in Table 15. Again, it must be remembered that our observations cover only a part of each day, and we have no means

TABLE 15
EFFECT OF TESTOSTERONE ON CROWING

Flock	Individual	Days In- jected before Crowing	Days Crow- ing Con- tinued after Injection	Daily Dosage (Milligrams)	Remarks
A 1, 2.....	{RG	38	3	1.0	Crowed over 60 times
	{BR	0.5	Not observed to crow
A 3, 4.....	{GG	45	0.5	Crowed 8 times on forty-fifth day; not heard later
	{BG	0.25	Not observed to crow
A 5.....	{BY	30	3	1.25	Crowed at least 74 times
	{RY	37	1.00	Crowed at least 20 times; not heard after injections ceased
	{BG	0.75	Not observed to crow (became ill)
B 2.....	{GG	16	1.25	Crowed 11 times by twenty-third day; not heard again (became ill)
	{RW	40	0.75-1.5	Crowed 17 times by fifty-eighth day; not heard again
	{BB	1.0	Not observed to crow (became ill)
C.....	{RR	28	0.75	Crowed at least 6 times
	{GG	34	0.75	Crowed at least 4 times

of knowing how much crowing went on when no observer was present. Eight of the 12 injected birds are known to have crowed, while none of the more numerous controls was heard to crow. The crowing did not begin until there had been over 2 weeks of daily injections, and in 5 cases is not known to have continued until the end of the period of injections.

RY of flock A 5 was heard to crow on the last day of injection; BY of the same flock and RG of flock A 1, 2 crowed on the third day following the cessation of treatment. After this particular BY and this RG began crowing, each was heard almost daily until 3 days after cessation of treatment. Their crowing seemed to increase in quality as time went on, until a fine loud call was produced, which was, however, never so deep or prolonged as were those given by neighboring cocks.

The data offer a decided suggestion that crowing is related to dosage of TP. In this connection the case of RW of flock B 2 is suggestive. This bird received originally 0.75 mg. daily and was first heard to crow on the fortieth day of injection; it crowed again

2 days later and then, so far as we know, was silent for 6 days. At that time her dosage was doubled, and almost immediately she crowed six times in succession.

The crowing, like changes in the social order, did not begin until there had been 2 or more weeks of treatment. Both phenomena occurred during the same general period; they were not, however, invariably associated, since in flock B 2 both **GG** and **RW** crowed actively but did not rise in the peck-order of the flock. On the other hand, 1 hen in each of the A flocks gained 1 or more social reversals over flock mates without being heard to crow.

Courtship.—In flock A 5, **BY** was three times seen to give a somewhat abbreviated exhibition of courtship. She would drop one wing and flutter it rapidly against her side while sidling up to another hen, and at that same time would move one foot up and down in an exaggerated manner. This behavior was a rather good reproduction of the courting reaction of a breeding cock. **BY** was seen to react so on the thirty-eighth, forty-sixth, and forty-ninth days of injection to **RW**, **RR**, and **RG**, respectively. **BY** had recently moved into the α -position of her flock; **RG** and **RW** were tied for second place (both pecked 6 others; **RG** pecked **RW**); and **RR** ranked eighth. **RW** of flock B 2 gave a similar reaction to the poulard **GY**. This was seen on the thirty-sixth day of injection.

Egg-laying.—Trap nests were present in the pens at all times, and the birds gradually learned to use them for laying; however, the trap-nest records were never perfect. **BR** of flock A 1, 2 had been laying steadily with a trap-nest record that averaged 0.52 eggs per day for the 81 days that preceded the beginning of TP treatment on June 6. She laid an egg on June 7 and thereafter did not lay in a trap nest until after August 15. Her hormone injections had stopped on July 29. **RG** of the same flock laid 0.4 eggs per day in the same preinjection period, ceased laying when injection began, and started laying again in late August. Each of the control hens in this flock continued to lay throughout these summer months at about the same rate they had shown in the late spring. They averaged 0.26 trap-nest eggs per hen per day before the injections started and 0.39 eggs during each of the 54 days of injection while each was receiving 0.1 cc. of sesame oil daily. There were 0.06 eggs per hen per day which could not be definitely allocated to a given hen in the preinjection period, and 0.05 per hen per day during the injection periods.

Flock A 3, 4 was also injected during June and July. Of the hormone-treated hens, **GG**, which received 0.5 mg. of TP daily, laid its last egg on the second day of injection; **BG**, which received half that amount, laid on days 2, 32, 35, 37, and 44. The controls in this flock laid fewer eggs during June and July than they had in the spring months. With the exception of **BR**, which became broody, each of the controls laid an occasional egg throughout the injection period. They laid 0.18 unassigned eggs per hen per day before, and 0.17 during, injections.

Because of molting, none of the hens in flock A 5 were laying regularly during the period of hormone treatment (August 30–October 19). Each of the control hens, with a single exception, laid once or more during that period; and one, **RG**, laid at least 27 eggs. **BG** and **RY** of the hormone-treated hens had laid one or more eggs in late August. **BG** is not known to have laid again before her death on October 31. Neither **RY** nor **BY**, the other hormone-treated hens in this flock, are known to have laid again until in January. They both began laying regularly in February. Only three “unassigned” eggs were laid in this flock during the injection period.

The pullets of flock B 2 had not learned to use the trap nests at the time their treatment began; hence, data from this flock are inconclusive. So far as they go, the records

support our previous experience that injection with TP greatly retards or prevents egg-laying. One of the pullets laid in the trap nests once only during the period of treatment, although each had done so previously. Three of their 5 pen mates that lived through the test period continued throughout to use the trap nests with some regularity. RW, the surviving hormone-injected bird of this flock, began laying actively in February about 6 weeks after treatment was stopped.

THE CASE OF YY

In connection with a different set of experiments we ran one more test on the effect of TP, which was barely completed when this manuscript was sent to press; hence, the results could not be readily distributed among the proper categories in the preceding pages. This has advantages since the case in hand summarizes many of the points already cov-

TABLE 16
THE SOCIAL ORDER IN FLOCK B 1, 2 BEFORE AND AFTER TREATMENT

A (APRIL 22)		B (JUNE 26)		
Individual	Number Pecked	Individual	Number Pecked	Formerly Pecked†
BW.....	9	YY.....	8	0
RW.....	6	BW.....	7	8
BY.....	6	BR.....	4	5
GY*.....	5	RW.....	4	5
BR.....	5	BY.....	4	5
GG*.....	4	GG*.....	3	3
RG.....	4	GY*.....	3	5
RY.....	3	RG.....	2	3
BG*.....	2	BG*.....	1	2
YY.....	1			

* Poulards; GG is well masculinized.

† RY died on June 13 and is not included.

ered and is, in many ways, a so-called "crucial" or "show" experiment, such as was more popular in an earlier period of experimental zoölogy.

Several deaths occurred among the highly inbred young Iowa birds of the B flocks during the winter. In early spring (March 24) the surviving members of flock B 2 were moved into the pen of the survivors of flock B 1. A month later they showed the peck-order which is listed in Table 16 (A). Beginning on April 22 YY was given 1.25 mg. of TP daily; BY and RY served as oil-injected controls. Injections were discontinued after July 9. It is of interest that RY, then called "RW" of flock B 2, had received 0.75 mg. daily from October 19 to December 4 and double that amount for the following 17 days and that she remained low in her social order.

YY was seen to begin revolting against her socially superior flock mates after 11 days of injection; 9 successful revolts were seen during May and early June. Her actual revolts began earlier, for after only 4 days of injections she revolted successfully and won a reversal over a sick hen, which later died and is not listed in Table 16. A week afterward she won her revolt against a sick poulard, which also is unlisted in the table. On May 7 YY defeated the poulards GY and BG, and on May 12 she won over BR. She

continued her upward climb in the flock order until on June 16 she ranked next to the top in number of subordinates, although she was still being pecked by her present despot (BW), by her former despot of flock B 2 (RG), and by the masculinized poulard GG. On June 17, YY rose over BW, on June 24, over GG, and became top bird of the flock on June 26 when she began pecking RG. YY has maintained this position to date (July 25).

Meantime the controls had been seen to give 2 unsuccessful revolts; and the masculinized poulard GG gave 2 reversals, 1 over a poulard (GY) and 1 over a sick bird. The effect of TP injections on the social position of YY is the more important, since, as shown on page 426, similar treatment when these particular birds were younger had not produced changes in the peck-order. At the beginning of the injections, YY was six months older than were the birds when the earlier treatment began; it was spring rather than autumn, with the difference in the egg-production cycle that this implies.

Other observed effects were all according to expectation and may be briefly summarized as follows:

1. The head furnishings became large and turgid, the face became very red, and the ear lobes became yellowish, as in cocks. The general carriage, especially of the tail, became more erect.

2. The size of the vent decreased almost to that typical of a cock, and the inter-pubic distance became less.

3. Spurs sprouted slightly.

4. No eggs were laid, however; YY was not laying just preceding treatment.

5. Crowing was first heard after 24 days of injections and continued steadily until 10 days after injection ceased and some 238 distinct crows had been recorded. During the last 7 days YY crowed only at dawn. Only one of our apparently normal hens has ever been seen to crow and this on only two occasions and in very rudimentary fashion. This individual laid an egg on one of the days she crowed. YY commenced calling hens to food like a cock on June 13 and was last noted to do this 3 days after stopping the injections.

6. YY began courting hens after 33 days of injection and a total of 86 such acts was recorded. Courtship was observed almost daily up to 7 days after the last injection, usually during initial encounters with strange hens. During several hundred such encounters only one apparently normal hen on only one occasion gave a reaction that could be said to resemble courtship.

7. YY won all 26 of her staged initial pair-contacts during the period of injection; all these were with normal hens. In previous series of such contacts she had lost except to poulards.

In the course of these staged encounters YY won one of the most severe combats we have yet seen between hens. She was introduced into a neutral pen with RY of flock D 1. RY is much heavier and older; has fairly high social status in her home flock; and is an experienced fighter, that usually wins. YY attacked, and the two fought vigorously for $21+7+2+2+12$ seconds in successive onsets. Each time YY was beaten and retreated, but she kept forcing the fighting after short rests. Finally she retreated to the roost, as losers often do, and RY scratched litter on the floor below, a reaction which winners frequently give.

After a few minutes the observer opened the pen door, and at that YY jumped down to the floor near RY, which at once leaped up and perched on a side bar some distance

above, while YY scratched about below. Then the latter jumped up to the roosts and from there courted RY, which was approximately on her level and some 30 inches away. YY crowed successively at short intervals, then leaped to the floor and crowed again. RY reacted as though frightened and, when again placed on the floor, flew immediately to the roost. YY followed and courted RY, which pecked her on the head. A brief fight ensued, which terminated when RY fell off the roost. She leaped immediately to the side bar and made strong avoiding reactions when YY approached.

The latter was then removed from the pen for 5 minutes until RY recovered enough to begin feeding, whereupon YY was slipped under the partly opened door and RY at once flew up to the side bar. YY was again removed and later was again introduced, with similar results; and still later RY avoided YY when the two were alone together in a part of RY's home pen. To all appearances YY won by not knowing when she was beaten. Such persistent aggressiveness has been seen only once among other hens during the course of our observations.

DISCUSSION

In order to begin to understand the foundations of the social order in flocks of the common domestic fowl, one must have data concerning two radically different aspects, namely, those factors which initially determine the position of a given individual in the peck-order and those which make for the maintenance of the position, once it is established. These two sets of factors are not necessarily mutually exclusive, although they do present contrasting emphases. The initial position in the social order is largely determined by the relative aggressiveness of the individuals which compose the flock. Maintenance of position is associated with the factors which make for social inertia; such factors as memory and habit reinforce, and may entirely replace, the aggressive behavior patterns which are so important in the origin of a social order in hens.

In the present experimental analysis we have been investigating primarily the physiological basis of aggressiveness which determines which of two hens or pullets will dominate in a first contact with a stranger from another flock. Also, after the social order is established, we have been particularly interested in analyzing the factors which produce successful revolts that lead to a change in the established order. Although we had indications at the beginning and have since then accumulated more evidence that psychological factors are involved in aggressiveness, we have been directly concerned with a more obviously physiological approach.

The presence of male hormone is a potential source of aggressiveness. Cocks are more aggressive than hens or capons, and cocks dominate hens in a heterosexual flock. Hens apparently secrete male as well as female hormone; that is, they appear to be functional endocrine hermaphrodites, since the combs of ovariectomized fowl drop to a capon level (Domm, 1927). In normal hens both the medulla, which secretes androgenic material that affects the comb, and the cortex, which secretes estrogenic substance that affects the oviduct, respond to gonadotropic agents (Domm, 1937). Hence, the introduction of additional male hormone means increasing the amount of some material originally present rather than the introduction of a wholly foreign substance. Our choice of testosterone propionate (TP) for injection was determined by its chemical purity, its known physiological action on the nervous system as well as on other organ systems (Bize and Moricard, 1937), its relative efficiency (Breneman, 1939), and its availability. The results obtained have justified the selection.

In three flocks of adult hens the TP-injected individual moved from at or near the bottom to the top of the social system, and each of the hormone-treated hens showed one or more social reversals over normal, healthy flock mates. Such a change was shown by none of the controls which were similarly injected with sesame oil. In one flock of relatively young, highly inbred pullets the treated birds did not rise in their social order within the flock but they did win 75 per cent of their first contacts with strange, untreated normal pullets and lost only to 2 aggressive, experienced fighters—and probably then because of illness. Six months later, one of these inbred birds gave an almost diagrammatic response to treatment with TP. Finally, with young pullets in which the peck-order was just being established, the 2 birds receiving TP became dominant in their respective flocks.

Although it is now generally considered that comb growth in capons, when it occurs, is a result of androgenic stimulation, the presence of the appropriate androgen in suitable quantity is not the only factor that determines the size of a fowl's comb. Different breeds vary in this characteristic as in others; even our different flocks of white leghorns had more or less definite variations in comb size. Within limits the amount of sunlight to which an individual is exposed affects the size of head furnishings (Domm, 1930). With these facts in mind, it is not surprising that the observed correlation between size of comb and aggressiveness in first combats was no higher than we found it.

The increased comb growth which we obtained was to be expected from the work of others (Hamilton, 1938; Breneman, 1938; Emmens, 1938), as was also the evidence of a decrease in the rate of egg-laying (Schoeller and Gehrke, 1933) and the lack of maintenance of normal rate of growth in young birds (Breneman, 1939). The greater weight of the older TP-treated hens is in keeping with the experience of Bulliard and Ravina (1938) with ducks about 2 months of age and is opposed to the usual result with growing chicks, our own included.

Our findings concerning the stimulation of crowing differ from those of Hamilton (1938) with very young female chicks and afford a demonstration of the conclusion reached by Myers (1917), and confirmed by Appel (1929), that there is no apparent anatomical reason why the female leghorn fowl should not crow, provided it has the instinct to do so properly developed. Incidentally, these observations throw some light on the question of the ontogenetic origin of instinctive behavior.

Benoit (1929, p. 380) found, by using testicular grafts, that the threshold of hormone stimulation in the cock ran as follows, with the last mentioned having the highest threshold: "(1) Epididyme et canal déferent; (2) Organes érectile; (3) Inhibition du plumage; (4) Instinct sexuel; (5) Chant; (6) Ardeur combative." Benoit suggested that changes which involve the intervention of the nervous system have a higher threshold than do changes in plumage or in comb and wattle growth.

In our experiments with females, changes in comb size were most readily and certainly effected; a change in the rate of egg-laying typically came next; the initiation of crowing, next; and finally changes in social position. It should be noted that in the last, and for our purpose the most important, aspect of these studies we have not investigated the dosage that will cause a given hen to become more aggressive in staged initial contacts; our data regarding dosage apply only to changes in the well-established peck-order of hens that had been living together for some time.

These observations that changes in behavior have a higher threshold than do changes in structure run counter to the early conclusions of ecologists (Shelford, 1911) that with

animals it is easier to effect modifications in behavior than in anatomy. We have at present nothing to say about the relative ease of producing changes in behavior and structure in the so-called "lower" animals; obviously, the generalization can no longer be applied to the vertebrates.

There are many indications of the complexity of the factors which initiate and maintain the social order among flocks of hens. One of these indications is found in behavior following the cessation of injections. The sequence of events was as follows: the comb began decreasing in size, courtship vanished, crowing ceased,⁷ and after a long interval egg-laying was resumed. In these respects the hens apparently returned to the *status quo ante*. However, the changes won in social position persisted as long as we followed the respective flocks—in the case of flock A 5, for at least 5 months.

Since social position in flocks of hens is not easily affected and changes come only after long treatment, this does not provide the physiologist with a satisfactory criterion for assay of hormone effectiveness. When the hormone begins to produce an increase in aggressiveness, the first revolts against social superiors are likely to fail even in cases when a few more injections will bring successful reversals. The first reversals shown by the low-ranking birds are likely to be the result of successes gained over those just above them in social rank and likewise relatively low in the social order. This is in part a result of the fact that birds near each other in social rank have more mutual contacts and in part because of the lesser aggressiveness one frequently finds in low-ranking hens. Only after a long period of injections, if at all, did our treated hens rise to the top of their respective flocks, and usually the last reversals were over the birds with the highest social status. It is entirely consistent with this picture that the social positions gained by the treated birds were maintained in the postinjection periods. The psychological factors which retarded her rise now helped maintain the hen in her newly acquired status.

It is fairly obvious, from what has been said, that the hen's relation to TP cannot be regarded as similar to that of chemicals in a test tube to which sufficient catalyst is added to cause the reaction to proceed to its logical conclusion. Among the other complicating factors are indications that recent experience in winning or losing encounters affects the outcome of the next pair-contact. These and other so-called "psychological factors" will be discussed in more detail in a later publication.

It is apparent that we have by no means solved the problem of the development and maintenance of social position even under the relatively simple conditions that exist in flocks of hens. We have shown that one physiological factor, the increase in the amount of male sex hormone present—at least to the extent that natural male hormone acts as does TP—if continued for some weeks, increases the likelihood that individuals so treated will better their position in the social order of the flock.

Our experiments were directed toward this one end, and we have not undertaken the arduous task of investigating the physiological mechanism(s) which finally produce changes. For that matter, we do not know with certainty the effect produced on an experienced hen by the physiognomy of a strange bird which possesses the high comb and general appearance of other birds which have recently held the peck-right over her. As an example, the behavior of BR₂ in contact with the large-combed but nonaggressive GG₁, as outlined in Table 13, suggests that this possible factor is not negligible.

⁷ Domm (1935) reports that the precocious crowing of male chicks stopped within 48 hours after the cessation of daily injections of hebin.

Finally, we have been considering some aspects of the social order in flocks of pullets and hens primarily because of our interest in the behavior and social life of birds. We have the distinct impression, however, that in working with this problem we are making an attack on some of the fundamental aspects of group living and graded social position as found in many vertebrates.

SUMMARY

1. Testosterone propionate (TP), injected into low-ranking individuals in these flocks of white leghorn hens, produced a rise in social status in each adult that was treated; and an injected individual eventually occupied the top position in each flock.

2. With one flock of younger pullets, in which the peck-order had just become fixed after an extended period of fluctuations, similar injections produced no changes in the social position within the flock. However, the hormone-treated pullets were more aggressive and successful in initial contacts with strange birds than they were before the injections.

3. In two flocks of battery-reared young pullets studied while the social order was beginning to be established, the injected birds came to dominate each flock.

4. In initial pair-contacts with strange birds, the hen or pullet with the larger comb had decidedly the better chance of dominating the situation; comb size is generally considered to be an index of the amount of male hormone present.

5. Other observed effects of TP include increased size of comb, retardation or suppression of egg-laying, initiation of crowing, and, with three hens, of courtship. These changes vanished soon after the cessation of treatment. Higher social position, once won, was retained.

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